

# Mutations in the *Cc.rmt1* gene encoding a putative protein arginine methyltransferase alter developmental programs in the basidiomycete *Coprinopsis cinerea*

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**Abstract** We characterized two developmental mutants of *Coprinopsis cinerea*, Apa56 and Sac29, newly isolated from a homokaryotic fruiting strain, 326 (*Amut Bmut pab1-1*), after restriction enzyme-mediated integration (REMI) mutagenesis. Both Apa56 and Sac29 exhibited slower mycelial growth than the parental wild-type strain and failed to initiate fruiting when grown on standard malt extract–yeast extract–glucose medium under 12 h light/12 h dark cycle. Both mutants exhibited unusual differentiation in aerial hyphae: differentiated hyphae lacked clamp connections and exhibited irregular shapes. The differentiated hyphae were similar to the component cells of hyphal knots, but did not form hyphal knots: they spread as dense mycelial mats. When the carbon source (glucose) in the medium was substituted with sucrose or galactose, both strains formed as many hyphal knots as the parental wild type. The hyphal knots formed, however, did not develop into fruiting-body initials, but developed into sclerotia. Molecular genetic analysis revealed that the gene, designated *Cc.rmt1*, is disrupted by REMI mutagenesis and is responsible for the phenotypes in both mutants. *Cc.rmt1* is predicted to encode a putative protein arginine methyltransferase, some homologs of which have been shown to

be involved in the regulation of gene expression in eukaryotes.

**Keywords** *Coprinopsis cinerea* · Mushroom · Protein arginine methyltransferase · Fruiting

## Introduction

Sexual reproduction in homobasidiomycetes is characterized by the formation of relatively large multicellular structures, fruiting bodies or mushrooms, for the efficient production and dispersal of basidiospores. In *Coprinopsis cinerea*, the developmental sequence of fruiting has been described in detail (Moore 1981; Kües 2000; Kamada 2002). Briefly, fruiting starts with the formation of hyphal aggregates less than 0.2 mm in diameter, called hyphal knots, on the mycelial colony of the dikaryon. Some of hyphal knots subsequently develop into fruiting-body initials with cap and stipe tissues differentiated in 1 day. The initials attain to fruiting-body primordia about 1 cm in height in a few days, which culminate in mature fruiting bodies with expanded caps bearing basidiospores and elongated stipes in one night.

To date, mutational analyses in *C. cinerea* have identified several genes that regulate various processes during fruiting-body formation. They include *cfs1* involved in the initiation of fruiting (Liu et al. 2006), *ich1* involved in differentiation of the cap tissue (Muraguchi and Kamada 1998), *eln2* (Muraguchi and Kamada 2000) and *eln3* (Arima et al. 2004) involved in stipe elongation, and *expl* involved in cap expansion and autolysis (Muraguchi et al. 2008). In other mushroom species than *C. cinerea*, on the other hand, gene expression analyses have identified a number of genes that show developmentally specific

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

Strain	Genotype/description	Source
326	<i>Amut Bmut pab1-1</i> /homokaryotic fruiting	P. J. Pukkila
KF <sub>3</sub> #2	<i>A91 B91</i>	This laboratory
Apa56	<i>Amut Bmut pab1-1 Cc.rmt1-1</i>	This study
Sac29	<i>Amut Bmut pab1-1 Cc.rmt1-2</i>	This study

expression (Leung et al. 2000; Miyazaki et al. 2005; Yamada et al. 2006; Joh et al. 2007; Chum et al. 2008). These gene expression studies suggest that several metabolic pathways and signal transduction cascades are involved in fruiting-body formation. However, molecular mechanisms regulating fruiting-body development in basidiomycetous mushrooms remain to be elucidated.

We have been isolating mutants defective in the initiation of fruiting from strain 326 (*Amut Bmut pab1-1*) of *C. cinerea* after restriction enzyme-mediated integration (REMI) mutagenesis. Strain 326 is a homokaryon carrying mutations in both *A* and *B* mating-type genes and forms fruiting bodies without the need of mating. In this study, we characterize two newly isolated REMI mutants, Apa56 and Sac29. Phenotypic analyses revealed that Apa56 and Sac29 exhibited slower mycelial growth than the parental wild-type strain and failed to initiate fruiting on standard malt extract–yeast extract–glucose medium. In both strains, the hyphae exhibited unusual differentiation: they were irregular in shape and lacked clamp connections such as the component cells of hyphal knots, but did not form hyphal knots. When glucose in the medium was substituted with sucrose or galactose, the mutant phenotypes were reduced and both mutants formed as many hyphal knots as the parental wild type. The hyphal knots formed, however, developed into sclerotia instead of fruiting-body initials. Molecular genetic analysis revealed that in both Apa56 and Sac29, the mutant phenotypes directly result from the disruption of a gene, designated *Cc.rmt1*, which encodes a protein showing high similarities to protein arginine methyltransferases.

## Materials and methods

### Strains, culture conditions and genetic techniques

Strains of *C. cinerea* used in this study are shown in Table 1. MY-glucose medium (1% malt extract, 0.4% yeast extract and 0.4% glucose) solidified with 2% (w/v) agar in 9 cm Petri dishes was used for routine mycelial cultures and fruiting. Slants of MY-glucose medium were used for fruiting. Plates of MY-sucrose medium (1% malt extract, 0.4% yeast extract and 1% sucrose) and

MY-galactose medium (1% malt extract, 0.4% yeast extract and 0.4% galactose) were also used for examination of mycelial growth and fruiting. Minimum (MM) medium was that of Shahriari and Casselton (1974) modified by Binninger et al. (1987). Cultures were maintained at 28°C under 12 h light/12 h dark cycle unless otherwise stated.

Genetic analyses were performed as described previously (Inada et al. 2001).

### REMI mutagenesis

REMI mutagenesis using plasmid pPHT1 (Cummings et al. 1999) was performed as described previously (Inada et al. 2001) except that *ApaI* or *SacI* was used as the restriction enzyme: *ApaI* and *SacI* were used for Apa56 and Sac29, respectively.

### Transformation

Transformation of *C. cinerea* was performed as described by Binninger et al. (1987) except that protoplasts were prepared from vegetative mycelia because the recipient strains, Apa56 and Sac29, did not produce sufficient oidia for protoplasts (data not shown). For mycelial protoplasts, vegetative mycelia grown on MY-glucose plates overlaid with cellophane were macerated in a Waring blender, incubated in MY-glucose liquid medium in a 9 cm Petri dish at 28°C for 2 days and then treated by lytic enzymes. Protoplasts released from the mycelia were purified by filtration through a sheet of gauze before use for transformation.

### Microscopy

Mycelia from the colonies of *C. cinerea* strains were harvested as described by Kamada and Tsuru (1993) for microscopic observation: briefly, a small piece of mycelia cultured on MY-glucose was excised from the inner part of the colony using a surgical knife and squashed between the slide glass and coverslip. Micrographs were collected by using a Zeiss Axiophoto microscope (Carl Zeiss AG, Germany).

### Plasmid rescue

Plasmid rescues were performed as described by Makino and Kamada (2004): briefly, the genomic DNAs of Apa65 and Sac29 were digested by *BamHI* and *EcoRI*, respectively. They were then self-ligated and introduced into *Escherichia coli* DH10B (Life Technologies, California, USA) cells. Plasmids were then isolated from the ampicillin-resistant transformants. Genomic DNAs from the *C. cinerea* strains were prepared as described by Zolan and Pukkila (1986).

**Table 2** PCR primers used in this study

Primer name	Sequence (5'-3')
<i>Cc.rmt1</i> forward 1	AATAGTGGATCCGGGGTTTCTGTGGG
<i>Cc.rmt1</i> forward 2	ACAGCTATGCCGATTCCTATGCCC
<i>Cc.rmt1</i> forward 3	AACCCCATCTCTCAAAGGAAAAACG
<i>Cc.rmt1</i> reverse 1	TCGCCGGATCCCCGTAAGACTTTCTC
<i>Cc.rmt1</i> reverse 2	TTGTAGGAGATGCCGATGTCGAGG
<i>Cc.rmt1</i> reverse 3	AGCGTGAGGTCCCGTTGAGAACTTG
AP-1	CCATCCTAATACGACTCACTATAGGGC
AP-2	ACTCACTATAGGGCTCGAGCGGC

### 5' - and 3' -RACE experiments

The *Cc.rmt1* cDNA was amplified from an existing cDNA library (Muraguchi and Kamada (1998) using two sets of nested gene-specific primers and the nested primers for the adaptors (AP-1 and AP-2) of cDNA clones (Table 2 and Fig. 2a). Amplified products were sequenced after cloning in pBluescript KS+ (Stratagene, La Jolla, CA).

### Vector construction

To construct a binary vector, designated pPAB2-rmt1, carrying the *C. cinerea pab1* and *Cc.rmt1* genes, a 3.5 kb genomic region containing the gene CC1G\_03287.2 predicted by the Broad Institute ([http://www.broadinstitute.org/annotation/genome/coprinus\\_cinereus/MultiHome.html](http://www.broadinstitute.org/annotation/genome/coprinus_cinereus/MultiHome.html)) was first amplified using the primers, *Cc.rmt1* forward 1 and *Cc.rmt1* reverse 1 (Table 2). The amplified product was digested with *Bam*HI and then inserted into the *Bam*HI site of plasmid pPAB2 carrying the *pab1* gene (Granado et al. 1997). Plasmid pPAB2-rmt1 was linearized by digestion with *Sph*I before use for complementation experiments.

## Results

### Isolation and genetic analysis of the REMI mutants, Apa56 and Sac29, defective in fruiting initiation

We newly isolated mutant strains, Apa56 and Sac29, defective in fruiting initiation among hygromycin-B resistant transformants of homokaryotic fruiting strain 326 (*Amut Bmut pab1-1*) after REMI mutagenesis. Apa56 was found among 1545 transformants after mutagenesis using restriction enzyme *Apa*I, while Sac29 was found among 673 transformants after mutagenesis using restriction enzyme *Sac*I. Both strains formed fruiting bodies normally when mated with a monokaryotic strain, KF<sub>3</sub>#2 (*A91 B91*), indicating that the mutations were both recessive. We then

analyzed the F<sub>1</sub> progenies from the matings and found that in both matings, the defect in fruiting cosegregated with hygromycin-B resistance in the four *Amut Bmut* progenies from the mating between Apa56 and KF<sub>3</sub>#2, three were hygromycin-B sensitive and normal in fruiting, and the remaining one was hygromycin-B resistant and defective in fruiting. Of the 14 progenies from the mating between Sac29 and KF<sub>3</sub>#2, seven were hygromycin-B sensitive and normal in fruiting and the remaining seven were hygromycin-B resistant and defective in fruiting. These results suggested that the mutations in Apa56 and Sac29 were both likely direct results of insertion of plasmid pPHT1 used for REMI.

### Phenotypes of Apa56 and Sac29

Under our standard culture conditions (MY-glucose plate 9 cm in diameter at 28°C under the 12 h light/12 h dark cycle), in the parental wild type inoculated at the center of the plate, the mycelial colony covered the whole surface of the plate and formed hyphal knots, the first visible structure of fruiting in a week. Some of the hyphal knots then underwent a series of developmental processes and formed mature fruiting bodies within 2 weeks after inoculation. Under the same culture conditions, strain Apa56 exhibited slower mycelial growth and formed a less fluffy and denser mycelial colony than the parental wild type (Fig. 1a). The mycelial colony formed did not produce a fruiting body after prolonged culture for a month. Microscopic observation revealed that in the mutants, most of the hyphae excised from the surface of the colony, except the marginal 1–2 mm region, differentiated into cells lacking clamp connections and exhibiting irregular shapes (Fig. 1b, panels 1 and 2) in contrast to slender hyphal cells with clamp connections from the inner parts of the colony of the parental wild type (Fig. 1b, panels 3 and 4). The marginal part of the mutant colony comprised normal vegetative hyphae with clamp connections (data not shown). The differentiated hyphae are like the component cells of hyphal knots, which are much shorter and thicker than vegetative hyphae and often have projections (Kamada and Tsuru 1993), but do not form a hyphal knot.

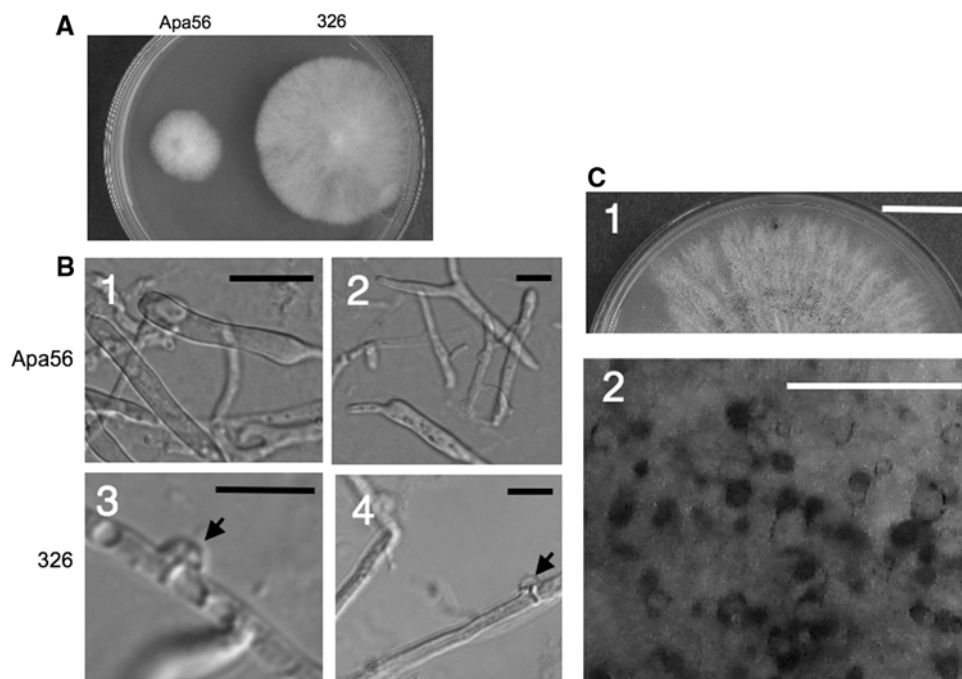
Colony formation and hyphal differentiation of another mutant strain, Sac29, were similar to those of Apa56 (data not shown).

### Carbon source influences hyphal differentiation of Apa56 and Sac29

Because it has been reported that fruiting initiation in *C. cinerea* is inhibited by glucose and glucose analogs (for a review, see Kües 2000), it is possible that the phenotypes of the mutants may be different on media with other carbon

**Fig. 1** Hyphal growth and differentiation in Apa56 and 326. **a** Mycelial colonies of Apa56 and 326 (parental wild type) 5 days after inoculation.

**b** Phase-contrast micrographs of aerial cells of Apa56 (panels 1 and 2) and 326 (panels 3 and 4). Note that the hyphae of Apa56 lack clamp cells. Arrows indicate clamp cells. Scale bar 10  $\mu$ m. **c** Panel 1 A colony of Apa56 cultured on MY-sucrose for 2 weeks. Scale bar 2 cm. Panel 2 Low magnification of the colony shown in panel 1, showing abundant sclerotia with pigmented rinds. Scale bar 1 mm



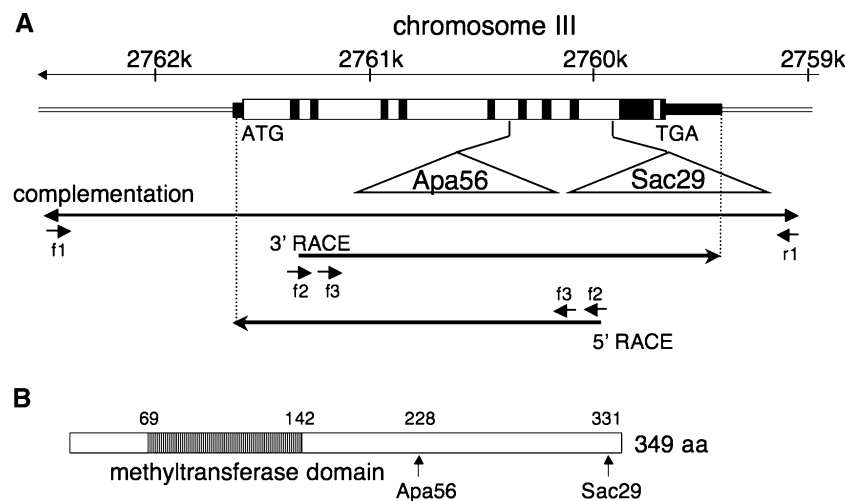
sources than glucose. To test this possibility, we substituted sucrose or galactose for glucose in MY-glucose medium. We found that on MY-sucrose or MY-galactose medium, strain Apa56 exhibited faster mycelial growth and formed a less dense mycelial colony and as many hyphal knots as the parental wild type. However, none of the hyphal knots formed developed into fruiting bodies, but many developed into sclerotia with pigmented rinds (Fig. 1c). Similar responses to the carbon sources were also observed in strain Sac29. In addition, the production of sclerotia in Apa56 and Sac29 was not affected by light (data not shown), although sclerotial production has been shown to be suppressed by light in an Amut Bmut strain (Boulianne et al. 2000). The parental wild type normally produced mature fruiting bodies on both MY-sucrose and MY-galactose media and produced no sclerotia (data not shown).

Gene disrupted in Apa56 and Sac29 encodes a putative protein arginine methyltransferase

On the basis of the results of genetic analysis described above, we performed plasmid rescues to identify the genes disrupted in the REMI mutants, Apa56 and Sac29. We could clone 9 kb and 2.5 kb genomic fragments adjacent to plasmid pPHT1 from strains Apa56 and Sac29, respectively. Partial sequencing of the genomic fragments suggested that pPHT1 is integrated within the hypothetical gene CC1G\_03287.2 predicted in the *Coprinopsis cinerea* (*Coprinus cinereus*) genome database ([http://www.broadinstitute.org/annotation/genome/coprinus\\_cinereus/](http://www.broadinstitute.org/annotation/genome/coprinus_cinereus/)

[MultiHome.html](#)) in both Apa56 and Sac29. PCR amplifications of the genomic DNA regions from the mutants followed by sequencing revealed that: in Apa56, a single copy of pPHT1 was inserted between 967 and 968 bp downstream of the translational start site of the predicted gene; in Sac29, a truncated copy of the plasmid was inserted 1,440 downstream of the translational start site and 10 bp just downstream of the insertion site was deleted (Fig. 2a).

To examine whether gene CC1G\_03287.2 complements the mutations in Apa56 and Sac29, we transformed these mutants with plasmid pPAB2-rmt1, which carries a 3.5 kb genomic fragment (supercontig 3: 2762382-2759026) ([http://www.broadinstitute.org/annotation/genome/coprinus\\_cinereus/MultiHome.html](http://www.broadinstitute.org/annotation/genome/coprinus_cinereus/MultiHome.html)) containing the whole length of the gene CC1G\_03287.2 and the *C. cinerea* *pab1* gene as a selectable marker. As described in “Materials and methods”, we transformed protoplasts prepared from vegetative mycelia in this study because the mutants did not produce a sufficient number of oidia for protoplasts (data not shown). We found that both defects (fruiting initiation and hyphal growth) of strain Apa56 were rescued by pPAB2-rmt1 in 53% (19/36) of *pab*<sup>+</sup> transformants. When we transformed Apa56 with plasmid pPAB2 carrying the *pab1* gene only as a control, the defects were not rescued (0/16). Plasmid pPAB2-rmt1 also rescued the defects of strain Sac29 [56% (9/16) of *pab*<sup>+</sup> transformants], while pPAB2 did not [0% (0/14)]. On the basis of these results, we conclude that the mutant phenotypes in Apa56 and Sac29 are direct results of insertion of pPHT1 within the predicted gene CC1G\_03287.2.



**Fig. 2** The *Cc.rmt1* gene disrupted in *Apa56* and *Sac29* encodes a putative protein arginine methyltransferase. **a** Structure of the *Cc.rmt1* gene. This gene is located on chromosome III (Supercontig 3 in the *C. cinerea* genome database). The *thick lines*, *open boxes* and *solid boxes* indicate the regions transcribed, a deduced ORF and the introns, respectively. *Vertical lines* connected to *triangular boxes* indicate the sites where plasmid pPHT1 was inserted in strains *Apa56* and *Sac29*. *Horizontal arrows* indicate the primers for 5'- and 3'-

RACE experiments and for the genomic fragment for complementation. **b** Predicted structure of the *Cc.Rmt1* protein. Pfam motif-finding software (Finn et al. 2008) indicated that the *Cc.Rmt1* protein contains a protein arginine methyltransferase domain at the N-terminal region (the *striped box*). *Vertical arrows* indicate the sites where the *Cc.Rmt1* protein is disrupted by pPHT1 in *Apa56* and *Sac29*, respectively

#### ORF determination of the *Cc.rmt1* gene disrupted in the mutants and characterization of the presumptive Cc.RMT

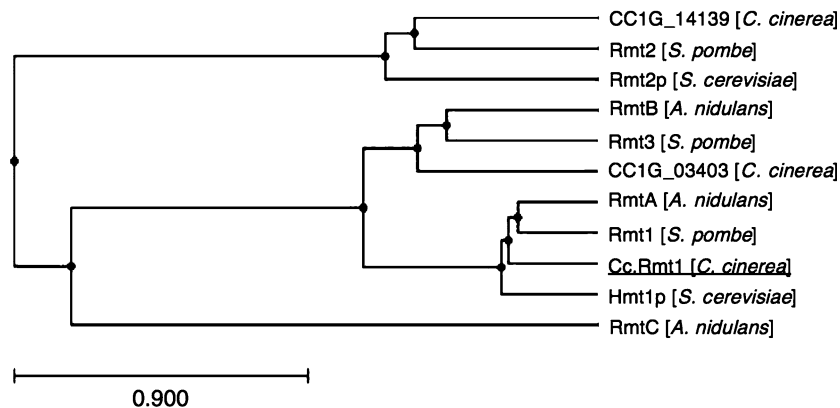
We performed 5'- and 3'-RACE experiments, sequenced cDNAs and compared the full-length cDNA sequence with the genomic DNA sequence from the *C. cinerea* database (URL is shown above), which identified an ORF interrupted by nine introns encoding a protein of 349 amino acids (Fig. 2). The full-length cDNA and deduced amino acid sequences have been deposited with the DDBJ/EMBL/GenBank data libraries under accession no. AB550661. The 5' splice sites agree with the consensus sequence GTRNGT found for filamentous fungi and the 3' splice sites with the consensus sequence YAG (Gurr et al. 1987), except that the first, second, third and sixth 5' splice sites are GTTGCG, GTGCGC, GTCCGT and GTAGGC, respectively. Blast searches revealed that the predicted protein had high similarities to protein arginine methyltransferases (Rmt) and hence the gene was designated *Cc.rmt1*. *Cc.Rmt1* is highly conserved in basidiomycetes: it exhibits the highest similarities to a *Laccaria bicolor* Rmt [NCBI Gene ID, 6073503 PRMT16202; score = 603, *E* value =  $1e-170$ , identities = 285/340 (83%)], a *Cryptococcus neoformans* var. *neoformans* Rmt (NCBI Gene ID, 3255710 CNBB2180) [score = 507; *E* value =  $7e-142$ , identities = 244/340 (71%)] and a *Ustilago maydis* hypothetical protein (NCBI Gene ID, 3634033

UM05900.1) [score = 501; *E* value =  $4e-140$ , identities = 238/327 (72%)].

Searches on the genome databases of *C. cinerea* (URL is shown above), the filamentous ascomycete *Aspergillus nidulans* ([http://www.broadinstitute.org/annotation/genome/aspergillus\\_group/MultiHome.html](http://www.broadinstitute.org/annotation/genome/aspergillus_group/MultiHome.html)), the fission yeast *Schizosaccharomyces pombe* ([http://www.sanger.ac.uk/Projects/S\\_pombe/](http://www.sanger.ac.uk/Projects/S_pombe/)) and the budding yeast *Saccharomyces cerevisiae* (<http://www.yeastgenome.org/>) indicated that *C. cinerea*, *A. nidulans*, and *S. pombe* have three genes for Rmts, while *S. cerevisiae* has two. Phylogenetic analysis of the predicted proteins by UPGMA (unweighted pair-group method with arithmetic mean) showed that *Cc.Rmt1* is the closest to *A. nidulans* RmtA, *S. pombe* Rmt1 and *S. cerevisiae* Hmt1p (Fig. 3).

#### Discussion

Here, we have characterized two newly isolated REMI mutants, *Apa56* and *Sac29*, defective in fruiting initiation. Both *Apa56* and *Sac29* exhibited slower mycelial growth than the parental wild type and did not form hyphal knots, the first visible sign of fruiting, under the culture conditions that normally promote fruiting, i.e. on MY-glucose medium and under 12 h light/12 h dark cycle. Hyphae in the inner parts of mycelial colonies of both mutants exhibited unusual differentiation as the colonies grew. The differentiated



**Fig. 3** A phylogenetic tree of Cc.Rmt1 and ten other fungal protein arginine methyltransferases in *S. pombe*, *S. cerevisiae*, *A. nidulans* and *C. cinerea* drawn by the UPGMA (unweighted pair-group method with arithmetic mean) using the CLC Free Workbench 4 software. The number shown in the figure is the distance matrix. Accession nos. that are not described in the text are as follows: *S. cerevisiae* Rmt2p (NCBI Gene ID, 3634033 NP\_009590.1), *S. pombe* Rmt2 (NCBI

Gene ID, 2542686 NP\_594160.2), *S. pombe* Rmt3 (NCBI Gene ID, 2541248 NP\_595572.1), *A. nidulans* RmtA (GenBank/EMBL/DBJ accession no. AY272042), *A. nidulans* RmtB (GenBank/EMBL/DBJ accession no. AY375304) and *A. nidulans* RmtC (GenBank/EMBL/DBJ accession no. AY375305). The amino acid sequences of hypothetical *C. cinerea* arginine methyltransferases are available from the *C. cinerea* genome database

hyphae lacked clamp connections and exhibited irregular shapes like the component cells of hyphal knots (Kamada and Tsuru 1993), but they spread as mycelia mats instead of forming distinctive hyphal knots. On MY-sucrose medium, in which the carbon source (glucose) in MY-glucose medium was substituted with sucrose, both mutants formed as many hyphal knots as the wild type. However, many of the hyphal knots developed into sclerotia and none of these developed into the fruiting-body initial even if light was given. Similar developmental processes were also observed when glucose in the medium was substituted with another carbohydrate, galactose. Because it has been reported that increasing glucose in the medium represses hyphal knot formation in an Amut Bmut strain of *C. cinerea* (Boulianne et al. 2000) and that light promotes the development of hyphal knots into fruiting-body initials and suppresses the development into sclerotia (Kües et al. 1998), both Apa56 and Sac29 might have altered the developmental responses to external signals such as carbon source and light. This hypothesis might be supported by the fact that the mutants produce much smaller number of oidia than the parental wild-type strain 326 under 12 h light/12 h dark cycle (data not shown), because oidia production is promoted by light in strains such as the parental wild type in which the A-regulated pathway is switched on (Kertesz-Chaloupková et al. 1998).

We found that gene *Cc.rmt1*, mutations of which are responsible for the phenotypes in both Apa56 and Sac29, encodes a putative protein arginine methyltransferase. Furthermore, we revealed that Cc.Rmt1 has high similarities to *A. nidulans* RmtA, *S. pombe* Rmt1 and *S. cerevisiae* Hmt1p. All of these four proteins exhibit significant sequence similarities to PRMT1, the predominant arginine

methyltransferase in mammalian cells (Bedford and Clarke 2009). Biochemical studies on *A. nidulans* arginine methyltransferases have revealed that RmtA methylates histone H4 (Trojer et al. 2004; Bauer et al. 2010). Histone H4 has also been shown to be a target of protein arginine methyltransferases CARM1 and PRMT1, which act as transcriptional coactivators for nuclear receptors in mammalian cells (Chen et al. 1999; Koh et al. 2001; Strahl et al. 2001). *S. cerevisiae* Hmt1 was shown to be involved in mRNA export into the cytoplasm for subsequent translation (McBride et al. 2000). *S. pombe* Rmt1 is involved in poly(A) tail length control (Perreault et al. 2007). Taken together, it is suggested that Cc.Rmt1 is involved in the control of gene expression via transcriptional and/or post-transcriptional regulations, which in turn suggests that fruiting-body initiation in *C. cinerea* is a complex process involving sophisticated regulation of gene expression. Future studies will include identification of the target protein(s) of Cc.Rmt1, which may provide information useful for the elucidation of molecular mechanism underlying developmental regulation in basidiomycetous mushrooms.

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