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Overexpression of a putative transcription factor *Gf.CRZ1* affects the expression of oxalate-degrading genes and causes morphological defects during mycelium formation in *Grifola frondosa*

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ABSTRACT

Grifola frondosa is commercially produced year-round in large-scale facilities. However, the molecular mechanisms of fruiting body differentiation remain elusive, and understanding these mechanisms at the molecular genetic level is therefore important for improving the cultivation of this economically important mushroom. In this study, we focused on a previously reported putative transcription factor, *Gf.CRZ1*, with homology to *Saccharomyces cerevisiae Crz1*. *Gf.CRZ1* is a candidate gene that is predicted to be involved in mutations affecting morphology and that is strongly expressed in some spontaneous mutant strains relative to wild type. Overexpression of *Gf.CRZ1* in the wild-type strain leads to increased expression of *Gf.ODC1* and *Gf.FDH1*, which are involved in oxalic acid degradation, similar to that of the mutant strains. Additionally, the *Gf.CRZ1*-overexpressing strains exhibited morphological defects including reduced colony growth rate and poorly developed aerial mycelia similar to mutant strains. These results suggested that overexpression of *Gf.CRZ1* might affect regulation of oxalic acid degradation and be causally related to morphological defects via disturbances in oxalic acid metabolism.

1. Introduction

Grifola frondosa (maitake) is a commercially important edible basidiomycete mushroom that is produced year-round in large-scale facilities. In commercial mushroom cultivation, optimization of cultivation conditions required for fruiting body development, including culture medium formulations, temperature, humidity, light, and other physical stimuli, is important for controlling quality and stabilizing production.

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However, conditions for mushroom cultivation are often based mainly on the cultivators' experience, because relatively little is known about how these conditions affect the efficiency of fruiting body development, let alone its underlying molecular mechanisms. A better understanding of mechanisms by which mushroom-forming fungi obtain the metabolites and nutrients required for vegetative growth and fruiting body differentiation could improve mushroom cultivation techniques.

In previous studies, we used microarrays to comprehensively analyze gene expression during the mushroom cultivation process (Kurahashi et al. 2012) and sequenced the whole genome of *G. frondosa* (Sato et al. 2013). Recently, we have reported analyses of microarray-based transcriptome comparisons between the wild-type strain Gf-N2 and fruiting body morphological mutant strains Gf-A1 and Gf-A4 to identify genes related to fruiting body differentiation or morphological mutations (Kurahashi et al. 2014). Strains Gf-A1 and Gf-A4 were isolated as spontaneous mutants during cultivation that exhibited phenotypic defects in fruiting body differentiation after primordia formation and pileus expansion, respectively. From this experiment, we have identified 24 genes that were expressed more than 2-fold higher in both Gf-A1 and Gf-A4 than in Gf-N2. Among these 24 genes, the only gene we identified that encodes a putative transcription factor was *Gf.CRZ1*, which is a homolog of *Crz1* in *Saccharomyces cerevisiae*. Crz1 is a transcription factor required for calcineurin-dependent transcriptional regulation of stress-responsive genes (Stathopoulos and Cyert 1997), and its nuclear localization and activity are regulated by calcineurin-mediated dephosphorylation (Stathopoulos-Gerontides et al. 1999). Crz1 includes a C2H2 zinc finger motif that binds to the calcineurin-dependent response element in the promoters of calcineurin-regulated genes (Stathopoulos and Cyert 1997), another motif that serves as a calcineurin docking site, and a serine-rich region required for calcineurin-mediated dephosphorylation (Stathopoulos-Gerontides et al. 1999; Boustany and Cyert 2002). Gf.CRZ1 is a protein of 345 amino acid residues with identity to known CRZ1 proteins in fungi, including *S. cerevisiae* (24%; NP_014371), *Aspergillus fumigatus* (23%; XP_750439), *Magnaporthe grisea* (28%; XP_359644), *Botrytis cinerea* (29%; XP_001561008), *Candida albicans* (23%; EAK97605), and *Cryptococcus neoformans* (33%; XP_566613). The sequence identities are mainly located in the C2H2 zinc finger domains in the C-terminal end. However, Gf.CRZ1 lacks sequence motifs similar to the calcineurin docking site and the serine-rich region in *S. cerevisiae* Crz1. During a previous transcriptome analysis, the expression levels of the homolog of calcineurin-regulated genes did not differ between Gf-N2 and mutant strains Gf-A1 and Gf-A4 that showed significantly higher expression of *Gf.CRZ1* (Kurahashi et al. 2014). Thus, *Gf.CRZ1* might not be involved in the calcineurin signaling pathway. Because *Gf.CRZ1* was much more strongly constitutively expressed in Gf-A1 and Gf-A4 than in Gf-N2, we asked whether the differences in the expression levels of this gene could contribute to the distinctive phenotype characteristics of these mutant strains.

In this study, we hypothesized that *Gf.CRZ1* is constitutively up-regulated in Gf-A1 and Gf-A4 during cultivation, which could affect the transcript levels of the other 23 genes that were previously identified as highly expressed in Gf-A1 and Gf-A4 compared with Gf-N2. In previous studies, we have established an efficient transformation system for *G. frondosa* that allows constitutive expression of transgenes (Sato et al. 2015). To elucidate the functional role of the *Gf.CRZ1*, we overexpressed *Gf.CRZ1* in strain Gf-N2 using our transformation system for *G. frondosa* and examined whether the expression levels of the other 23 identified genes were increased by up-regulation of *Gf.CRZ1*. When the expression levels of some of the 23 genes were analyzed, the expression of *Gf.FDH1* (AB830707), encoding for an NAD-dependent formate dehydrogenase (FDH, EC 1.2.1.2), was increased upon up-regulation of *Gf.CRZ1*. The up-regulation of *Gf.CRZ1* also enhanced the expression of *Gf.ODC1* (AB981589), encoding for oxalate decarboxylase (ODC, EC 4.1.1.2), an enzyme that catalyzes the step preceding catalysis of oxidation of formate to CO_2 by FDH in the oxalate metabolism pathway. These results suggest that *Gf.CRZ1* is likely involved in regulation of the expression of *Gf.FDH1* and *Gf.ODC1*, which are responsible for oxalic acid degradation. In white-rot fungi, including *G. frondosa*, regulation of intra-and extracellular oxalic acid levels has been thought to contribute to lignin degradation (Kuan and Tien 1993; Urzúa et al. 1998; Lundell et al. 2010). Additionally, we show that overexpression of the *Gf.CRZ1* leads to phenotypic changes similar to those observed in the vegetative mycelia of mutant strains Gf-A1 and Gf-A4.

2. Materials and methods

2.1. Strains and culture conditions

G. frondosa dikaryotic strain Gf-N2, an isolate derived originally from commercial strain M51, was used as the wild-type strain in this study. Fruiting body mutant strains Gf-A1 and Gf-A4 are spontaneous mutants described in our previous study (Kurahashi et al. 2014) and were compared in these experiments. Strains were maintained on potato dextrose agar (PDA; Oxoid, Basingstoke, UK) in darkness at 25 °C.

2.2. Construction of Gf.CRZ1 expression vector

To express *Gf.CRZ1* constitutively, a *Gf.CRZ1* expression vector was constructed using the promoter and terminator of the translation elongation factor 3 gene (*Gf.TEF3*: AB909029) for strong constitutive expression. The coding region of *Gf.CRZ1* was amplified by PCR from the subcloned full-length cDNA of *Gf.CRZ1* as a template using primers Gf.CRZ1-F and Gf.CRZ1-R. The promoter and terminator regions of *Gf.TEF3* were amplified by PCR from pGFtef3-hph (AB909455) using primer pairs Ptef3-F2/Ptef3-R and Ttef3-F/Ttef3-R2, respectively. The primers used for amplifications of these fragments contain 15-base extensions at the 5′ ends required for the In-Fusion® HD Cloning system (Clontech, Mountain View, CA, USA) and are listed in Table 1. The amplified fragments of the *Gf.TEF3* promoter, *Gf.CRZ1* coding sequence, and the *Gf.TEF3* terminator were ligated using the In-Fusion cloning system in the stated order into *Hin*dIII- and *Sal*I-digested pGFgapdh-hph (AB909452). The pGFgapdh-hph vector contains the bacterial hygromycin B phosphotransferase gene (*hph*) driven by the