Full Paper

Expression specificity of *CBP1* is regulated by transcriptional repression during vegetative growth of *Magnaporthe oryzae*

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The rice blast fungus *Magnaporthe oryzae* produces appressoria during the infection of a host. In *M. oryzae*, the appressorium formation-related gene *CBP1* (*Chitin Binding Protein 1*) is specifically expressed during the early stage of appressorium differentiation. The transcription factor *CON7* activates *CBP1* expression. However, many aspects of the regulation of *CBP1* expression are still unknown. In this report, the *CBP1* 5' upstream region was analyzed using an *egfp* reporter. Deletion of the *CBP1* 5' upstream region caused derepression of reporter gene activity during vegetative growth. This result suggests that *CBP1* expression is repressed during vegetative growth. The key 5' upstream sequences for *CBP1* repression were examined. Furthermore, *cis*- and *trans*-acting elements of the negative regulatory region were investigated. Here, we discuss the transcriptional regulatory mechanism of *CBP1*.

Key Words—*CBP1*; *Magnaporthe oryzae*; transcriptional repression

Introduction

The rice blast fungus *Magnaporthe oryzae* (Couch and Kohn., 2002) is a heterothallic ascomycete that causes rice blast disease. The conidium of this fungus elongates a germ tube after adhesion to the host surface. The germ tube senses surface hardness, hydrophobicity, and chemical components, such as soluble cutin monomers and lipid monomers (Dean, 1997; Gilbert et al., 1996; Xiao et al., 1994). This surface sensing generates cues for differentiation. The fungus

differentiates a specialized infection structure called an appressorium from the tip of the germ tube. A penetration peg from the appressorium penetrates the host cuticle and breaks into the host cells (Howard et al., 1991). In the chain of this infectious process, at least two signal transduction pathways are known to be involved. One is a cyclic AMP (cAMP)-mediated pathway, and the other is a MAP kinase signaling pathway. The cAMP-mediated pathway is required for appressorium formation on hydrophobic surfaces (Dean, 1997), and the MAP kinase signaling pathway is required for appressorium formation and pathogenic development (Xu and Hamer, 1996).

In *M. oryzae*, the gene *CBP1* was identified (Kamakura et al., 2002). *CBP1* encodes a putative extracellular chitin deacetylase-like protein and contains an extra chitin binding domain, and a $\Delta cbp1$ strain

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showed a dramatic reduction in appressorium differentiation only on artificial substrates. Interestingly, *CBP1* is expressed in the germ tube (early stage of appressorium differentiation), but the mRNA of *CBP1* could not be detected in vegetative hyphae even using reverse transcription polymerase chain reaction (RT-PCR) (Kamakura et al., 2002). These phenomena indicated that the *CBP1* product is involved in appressorium differentiation, and *CBP1* expression is appressorium specific.

Transcriptional regulation plays a key role in controlling the growth and differentiation of eukaryotic cells. Therefore, analysis of the mechanism of transcriptional regulation of genes expressed specifically during the differentiation stage of appressoria will be important for understanding the mechanism of appressorium morphogenesis. In a previous report, a transcription factor, *CON7*, of *M. oryzae* was identified as a central regulator of appressorium differentiation (Odenbach et al., 2007). The transcriptional activation of *CBP1* during germ tube elongation was found to depend on *CON7*. Although *CON7* regulates numerous genes, the regulatory mechanisms of individual regulated genes are still unclear.

In this report, we focused on the transcriptional regulation of *CBP1*. We tried to elucidate fundamental information about the mechanism of transcriptional regulation of *CBP1* using routine techniques. As a result, we found evidence for the negative regulation of *CBP1* during vegetative growth. Herein, we also discuss important questions concerning the regulation mechanism that allows the switching of *CBP1* expression and the importance of negative regulation of gene expression for differentiation of *M. oryzae*.

Materials and Methods

Fungal strains and culture conditions. *Magnaporthe oryzae* strain P2, a Japanese rice-pathogenic isolate, was used throughout this study. Cultures were maintained on potato dextrose agar (PDA; Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) and on oatmeal agar (Difco Laboratories, Sparks, MD, USA) at 28°C. For conidiation, this fungus was inoculated onto oatmeal agar plates and cultured for 2 weeks. After removing aerial mycelia with a sterilized brush, conidiation was induced by placing the oatmeal agar plates under BLB lamps (FL20S, 20W; Toshiba Co., Ltd., Tokyo, Japan). Conidia were harvested in sterile water.

Transformation of *M. oryzae*. Production of protoplasts of *M. oryzae* was performed as described previously (Kimura et al., 1995). Protoplasts were washed with STC buffer (1.2 M sorbitol, 20 mM CaCl₂, 10 mM Tris-HCl, pH 8.0), and were prepared at a concentration of 10⁸ cells/ml in STC buffer. DNA solution (2 µl at approximately 100 ng/µl) was gently added to 20 µl of protoplast suspension. PEG solution (25 µl; 60% PEG4000; Kanto Chemical, Co., Inc., Tokyo, Japan), 50 mM CaCl₂, 10 mM Tris-HCl, pH 8.0) was gently added to the protoplast mixture twice. After incubation for 20 min, 1 ml of STC-50 buffer (1.2 M sorbitol, 50 mM CaCl₂, 10 mM Tris-HCl, pH 8.0) was added and gently mixed. Protoplasts were collected by centrifugation (3,000 × *g* at room temperature for 5 min) and suspended in YG-1/2SC medium (0.5% yeast extract, 2% glucose, 0.6 M sorbitol, 25 mM CaCl₂). The protoplast mixture was incubated at room temperature for 3 h to promote expression of the antibiotic resistance gene. After incubation, 200 ml of this mixture was suspended in YG20S agar medium (0.5% yeast extract, 2% glucose, 20% sucrose, 1.5% agarose) containing Hygromycin B (Wako Pure Chemical Industries, Co., Ltd., Osaka, Japan; 100 µg/ml) or blasticidin S (Kaken Pharmaceutical, Tokyo, Japan; 50 µg/ml). Plates were incubated at 28°C for 2 or 3 days and YG agar medium (0.5% yeast extract, 2% glucose, 1.5% agarose) containing hygromycin B (150 µg/ml) or blasticidin S (75 µg/ml) was overlaid. After incubation at 28°C for 3 days, mycelial colonies that grew through the overlaying medium were transferred to PDA medium.

Construction of plasmid vectors. An outline of the plasmid constructs used in this study is shown in Fig. 1. A 2.8 kb *HindIII*-*XhoI* fragment containing the *CBP1* 5' flanking region from the cosmid vector (Kamakura et al., 2002) was cloned into pBluescript SK+ (Stratagene, La Jolla, CA) and named pCBP1L. A *CBP1* 5' upstream fragment consisting of the region from the *HindIII* site to the start codon was obtained from pCBP1L. This fragment was directly connected to the start codon of the *egfp* gene. This construct was truncated using the *PstI*, *Clal*, and *NdeI* sites located in the *CBP1* 5' upstream region. Each construct was inserted into the *EcoRV* site of a pCSN43 plasmid vector (Staben et al., 1989) in the same direction (Fig. 1). The resulting plasmids carrying the *egfp* gene driven by the *CBP1* 5' upstream region up to *HindIII*, *PstI*, *Clal*, and *NdeI* site were named pPCGHd, pPCGPt, pPCGCI, and pPCGNd, respectively. *CBP1* 5' upstream frag-

Fig. 1. Outline map of the plasmid vector carrying the reporter constructs.

The plasmid vectors carrying the reporter constructs were based on the plasmid vector pCSN43 (*hph*: Hygromycin B phosphotransferase, *bla*: beta-lactamase). The 5' upstream region of *CBP1* was directly connected to the start codon of coding sequence of *egfp*. This construct was introduced in a fixed direction into pCSN43 at the *EcoRV* site. All reporter constructs used in this study were introduced into pCSN43 in the direction shown in this figure.

ments truncated by the about 50 bp were prepared by polymerase chain reaction (PCR). Amplified fragments were used to replace the corresponding region of pP-CGCl. An internal deletion mutation of the *CBP1* 5' upstream fragment was prepared by the overlap extension (SOE) method (Horton et al., 1989).

To create the *MoHAPB* disruption vector pDHB, a cosmid vector containing a 10 kb *XhoI* fragment including the *MoHAPB* locus was isolated from a cosmid library of *M. oryzae* P2 strain (Kamakura et al., 1999) by colony hybridization. A digoxigenin (DIG) labeled *MoHAPB* probe was prepared using primer pair MGHB2_S (5'-CTACAGCTTACCGTCGGA-3')/MGHB_AS (5'-TCATCCATCGTCCTCGGCATCCT-3') by the DIG PCR labeling procedure in accordance with the manufacturer's protocol (Roche Applied Science, Indianapolis, IN, USA). The 10 kb *XhoI* fragment was cloned and the resulting plasmid was named pHBX. The 1.1 kb *blasticidin S deaminase* gene (*BSD*) cassette was amplified using primer pair BCA (5'-CCGTA CGAGGTGCAGAGAAGATGA-3')/BCAS (5'-TCGTACG AGAAGGCACTCTTTGCTGCT-3') (an additional *BsiWI* site is underlined) from template vector PBF101MBD (Kamakura et al., 2002) and inserted into the *BsiWI* site of pHBX.

DNA manipulation and Northern blot analysis. We followed standard procedures for manipulations such as plasmid DNA isolation, restriction enzyme digestion, and hybridization (Sambrook et al., 1989).

Visualization of *egfp* expression in the appressorium

formation stage and vegetative hyphae. Conidia of *M. oryzae* were suspended in sterile water at a concentration of less than 10^5 /ml, and 20 μ l of this suspension was dropped on a micro coverglass (MATSUNAMI GLASS Ind., Ltd., Osaka, Japan). This micro coverglass was placed gently at room temperature and in humid conditions for 6 h. After incubation, samples were observed using a fluorescence microscope (DM5000; Leica Microsystems GmbH, Wetzlar, Germany). Samples were excited and viewed using an L5 filter set. Sample images were taken using Leica IM version 4 software.

For the observation of fresh vegetative hyphae, minuscule pieces of hyphal tips were acquired from the tip of mycelia on PDA plates using a pipette tip and inoculated into 1 ml of YG medium in a 24 well test plate. After an incubation of 24–30 h at 28°C, grown hyphae were acquired and washed with sterilized H₂O. *egfp* expression was then observed using a fluorescence microscope as described above.

RNA isolation and RT-PCR. For preparation of total RNA during vegetative growth of *M. oryzae*, vegetative hyphae were frozen by liquid nitrogen. Total RNA of vegetative hyphae was extracted using ISOGEN (Nippon Gene Co., Ltd., Toyama, Japan) in accordance with the manufacturer's instructions. For the preparation of total RNA of germinated conidia, a conidial suspension (approximately 10^5 /ml) in distilled water was dropped onto a hydrophobic polycarbonate plate. The plate was incubated at room temperature in humid conditions. After germination, the plates were frozen in liquid nitrogen and freeze-dried. The freeze-dried germinated conidia were scraped from the plate using the edge of a razor. Total RNA of germinated conidia was extracted from the collected samples using ISOGEN. The resulting total RNA was reverse transcribed using a Ready-To-Go you prime first strand kit (GE Healthcare, Chalfont, UK) with oligo (dT)₁₂₋₁₈ primer (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer's instructions. RT-PCR was conducted with primer pairs PK3F_U (5'-ACGGTGACCACGCCATCCC C-3')/A4-L (5'-CGAAGTAGATGGCATGATAACCAAGC TC-3') and MGBTUB_S (5'-TGGTTGGCTTCGCTCCTT TGAC-3')/ MGBTUB_AS (5'-GAACTGCTCACCAGACA CGCTTG-3') for the detection of *CBP1* and *beta-tubulin* transcripts, respectively.

Results

Transcriptional regulation of CBP1 is under the influence of its 5' upstream region

To monitor expression of *CBP1*, the *CBP1* 5' upstream region (containing the region from the translation start codon to the *Hind*III recognition site; see Fig. 2, top bar) was cloned and connected directly to the translation start site of a reporter gene, *egfp*. The resulting reporter construct was inserted into pCSN43 (Fig. 1). This reporter plasmid was introduced to *M. oryzae* strain P2 by protoplast PEG-mediated transfor-

mation. After transformation, hygromycin B-resistant transformants were selected. In the resulting transformants, the insertion of the full-length reporter construct into the genomic DNA was confirmed by PCR, and ectopic insertion of the reporter construct into the genomic DNA was detected by DNA gel blot analysis (data not shown). After this, a screen was performed for transformants containing various reporter constructs using the same methods.

The *egfp* expression in the resulting transformants was examined by fluorescence microscopy. During vegetative growth, the green fluorescence intensity

Fig. 2. Expression profiles of *egfp* under control of the truncated *CBP1* 5' upstream regions.

(A) Images obtained using fluorescence microscopy of hyphae and appressoria are shown. A diagram of the reporter constructs is shown on the left side, and images showing *egfp* expression in each transformant are in the right-hand panels. Transformants were obtained for four independent clones for each construct, and representative images are shown in this figure. The top bar indicates the authentic *CBP1* 5' upstream region. The restriction enzyme recognition sites are indicated: H, *Hind*III; P, *Pst*I; C, *Clal*; N, *Nde*I. Scale bar = 10 μ m. (B) Northern blot analysis of *egfp* transcription in vegetative hypha of each transformant or wild type strain P2. Transcripts of *egfp* or *beta-tubulin* (MGG_00604.6) were detected. A photograph of the ethidium bromide-stained RNA used for Northern blot analysis is shown at the bottom.

level of each transformant had the same level of non-specific self fluorescence as the P2 strain. So we concluded that *egfp* expression was not detected in the hyphae of the transformants. On the other hand, *egfp* expression was clearly detected at the appressorium formation stage (Fig. 2A, *HindIII*). This result indicated that *egfp* was expressed in the transformants in the same pattern as that of *CBP1*. Furthermore, because the reporter construct was inserted into ectopic sites in the genomic DNA, this result also indicated that the transcriptional regulation capacity of the *CBP1* 5' upstream region did not depend on the authentic *CBP1* genomic locus. Thus, it was suggested that the transcriptional regulation of *CBP1* is under the influence of the 5' upstream region of the gene.

The CBP1 5' upstream region contains a negative regulatory region that functions in vegetative hyphae

The *CBP1* upstream region contains several restriction enzyme recognition sites, specifically *PstI*, *Clal*, and *NdeI* sites (Fig. 2A, top bar). We prepared truncated constructs consisting of *egfp* driven by the *CBP1* 5' upstream region up to each restriction enzyme recognition site. These truncated constructs were introduced to the *M. oryzae* strain P2, as described above. For transcriptional activity analysis, four independent transformants for each plasmid vector were obtained.

The *egfp* expression activities in these transformants were estimated by fluorescent microscopy (Fig. 2A). Green fluorescence of *egfp* was observed in appressoria at the differentiation stage of all transformants. In transformants harboring constructs extending up to the *PstI* and *Clal* recognition sites, the *egfp* fluorescence intensity in vegetative hyphae was extremely low. On the other hand, in transformants containing the *CBP1* upstream region up to the *NdeI* recognition site, the *egfp* fluorescence intensity in vegetative hyphae was apparently higher (Fig. 2A). This result indicated the expression of *egfp* in vegetative hyphae. Moreover, this result provided evidence for the negative regulation of *CBP1* during vegetative growth.

We also measured the transcriptional level of *egfp* in vegetative hyphae of these transformants by Northern blot analysis (Fig. 2B). Transcripts of *egfp* or *beta-tubulin* were detected by a specific probe for each gene. The result was consistent with the result of microscopic fluorescence analysis of *egfp*. However, in transformants harboring the construct extending up to the *Clal*

site, a very small amount of *egfp* transcripts was detected (Fig. 2B). This transcriptional level of *egfp* was clearly lower than that of the transformant by the construct extending up to the *NdeI* site, and suggested that the transcription of *egfp* of this construct was significantly repressed. Therefore, we inferred that the region between the *Clal* recognition site and the *NdeI* recognition site of the *CBP1* 5' upstream region is probably important for negative regulation in vegetative hyphae. After this, in microscopic fluorescence analysis, we used the expression level of the construct extending up to the *Clal* site as the threshold of negatively regulated expression.

Identification of the 5' negative regulatory region of CBP1

As the next step in the analysis, we tried to define the key sequences required for the transcriptional repression of *CBP1* in vegetative hyphae. We produced more detailed truncated deletion constructs sets of the 5' regulatory region of *CBP1*. These deletion mutants of the *CBP1* 5' upstream region were designed to be truncated in approximately 50 bp steps from the 5' end. Each of the plasmid vectors carrying *egfp* driven by these eight deleted fragments was introduced into *M. oryzae* strain P2. Each transformant was confirmed to contain the correct construct by PCR (data not shown). The appressorium differentiating stage and vegetative hyphae of the transformants were observed by fluorescence microscopy (Fig. 3A).

In transformants with constructs containing up to -1009 to -806 bp from the *CBP1* translation start site, *egfp* fluorescence in vegetative hyphae was not detected. However, weak *egfp* fluorescence was detected in hyphae of a few transformants with constructs containing up to -857 or -806 bp from the translation start site. Because distinct *egfp* fluorescence in the hyphae was not detected in the majority of transformants, we speculated that this weak expression of *egfp* was caused by positional effects of the insertion site of the reporter construct into the genomic DNA. On the other hand, in transformants with constructs containing up to -758 to -664 bp from the translation start site, *egfp* fluorescence intensity in vegetative hyphae was clearly observed. We designated this region between -806 and -759 bp from the translation start site of *CBP1* as NRS1 (Negative Regulatory Sequence 1). We hypothesized that NRS1 was important for the transcriptional repression of *CBP1* in vegetative hyphae.

Fig. 3. Identification of 5' negative regulatory sequence of *CBP1*. (A) The expression of *egfp* affected by 5' deletion or internal deletion of *CBP1* regulatory region. The pattern diagram of 5' deletion or internal deletion of *CBP1* regulatory region is shown at the left side. The *egfp* was connected to these fragments and introduced to the fungal genome. The *egfp* expression was observed by the detection of fluorescence. The top bar indicates the authentic *CBP1* 5' upstream region. ATG shows the translation initiation codon of *CBP1*. The restriction enzyme sites are marked: C, *Cla*I; N, *Nde*I. (B) The nucleotide sequence of NRS1 is shown. A known repressor binding sequence was not detected. An interesting sequence, CCAAT, is underlined.

To confirm this hypothesis, we prepared a construct containing an internal deletion of NRS1 (Fig. 3A, bottom bar), and introduced this into the *M. oryzae* strain P2. In transformants containing this construct, the expression of *egfp* in vegetative hyphae was confirmed (Fig. 3A). This result strongly supported our hypothesis.

Cis- and trans-acting element of NRS1

The nucleotide sequence of NRS1 is presented in Fig. 3B. We searched for *cis*-acting elements in NRS1 using the TRANSFAC database (Wingender et al., 1997). However, there were no consensus sequences known to bind repressive factors. However, NRS1 contained one interesting *cis*-element-like sequence, CCAAT (CCAAT-box-like sequence, underlined)(Fig. 3B). The CCAAT-box is often found in eukaryotic promoters (Brakhage et al., 1999; Edwards et al., 1998; Mantovani, 1999) and is widely recognized as a *cis*-acting element for the activation of gene expression. In the filamentous fungus *Aspergillus nidulans*, the CCAAT-binding complex AnCF has been studied extensively (Kato, 2005). AnCF contains at least three subunits,

HapB, HapC, and HapE. HapB is known as an important subunit of the CCAAT-binding complex of *A. nidulans*. It was shown that a single HapB subunit transports other subunits to the nucleus via a "piggyback mechanism" (Steidl et al., 2004).

Interestingly, in *A. nidulans*, there are examples that suggest a negative regulatory function of the CCAAT binding complex (Steidl et al., 2001; Weidner et al., 2001). Therefore, there is a possibility that the CCAAT sequence in the NRS1 and the CCAAT-binding complex of *M. oryzae* regulate *CBP1* expression repressively in vegetative hyphae. To test this hypothesis, we introduced *egfp* controlled by the *CBP1* 5' upstream region (up to -1009 bp from translation start site) with the CCAAT sequence deleted from the NRS1 into *M. oryzae* strain P2, but *egfp* expression was not confirmed in the vegetative hyphae (data not shown). Furthermore, we also demonstrated gene disruption of the HapB homologue gene (*MGG_00354.6*) based on insertional mutagenesis. Here, we designated *MGG_00354.6* as *MoHAPB*. Gene disruption of *MoHAPB* was performed by homologous recombination (Fig. 4A, B). The resulting *MoHAPB* disruptant of *M.*

Fig. 4. Effect of *MoHAPB* on the transcriptional repression of *CBP1* during vegetative growth.

(A) Restriction map of a 10.0 kb *XhoI* fragment of the *MoHAPB* locus is shown. A 1.1 kb *BsiWI* fragment containing the blasticidin S deaminase gene (*BSD*) cassette was inserted into a *BsiWI* site located in the *MoHAPB* open reading frame to create a gene disruption vector, pDHB. *MoHAPB* was disrupted by homologous recombination. (B) DNA gel blot analysis of a $\Delta MoHAPB$ mutant with a probe specific for *MoHAPB*. Genomic DNA was digested with *NdeI* (N) or *BglII* (B) and hybridized with the *MoHAPB*-specific probe shown as a gray box in A. (C) Transcription of *CBP1* during vegetative growth. The results from RT-PCR analyses of wild-type strain P2 and the $\Delta MoHAPB$ mutant are shown. *Beta-tubulin* from *M. oryzae* was used as a transcriptional control gene. VH, vegetative hyphae; GT, germ tube (early stage of appressorium formation).

oryzae showed a dramatic reduction in vegetative growth rate and conidiation, and these phenotypic defects were rescued by transformation of the mutant with a wild-type DNA fragment containing the 5' upstream region and open reading frame of *MoHAPB* (data not shown). However, in the *MoHAPB* disruption mutant, *CBP1* transcription during vegetative hyphae was still strictly repressed (Fig. 4C). Hence, we inferred that the CCAAT sequence in *NRS1* and *MoHAPB* do not have an essential role for the strict repression of *CBP1* in vegetative hyphae, and that a further unknown element(s) is involved in vegetative repression of *CBP1*.

Discussion

Using *egfp* reporter analysis, we investigated the regulation of *CBP1* expression under the influence of

CBP1 5' upstream sequences. In this report, reporter constructs were inserted into ectopic loci in the genomic DNA, and we also investigated whether the regulatory activity of the *CBP1* 5' upstream region is independent of the authentic genomic locus. These features enabled us to analyze the regulation activity of various truncated *CBP1* 5' upstream regions.

The expression of *egfp* driven by a truncated *CBP1* 5' upstream region (up to the *NdeI* site) was observed in vegetative hyphae. This result indicated the loss of expression specificity. The various truncation analyses of *CBP1* 5' upstream regions also showed distinct evidence for the vegetative repression of *CBP1*. In a previous report, the expression of *CON7* was not detected in vegetative hyphae (Odenbach et al., 2007). Therefore, it is reasonable conclusion that the expression of *egfp* driven by a truncated *CBP1* 5' upstream region during vegetative hyphae occurred in the ab-

sence of the *CON7* gene product. Furthermore, it was also reported that the transcription of *CBP1* in the germ tube of a Δ *CON7* mutant was decreased but did not disappear completely (Odenbach et al., 2007). These previous findings and the present results strongly suggested that *CON7* was not essential for the initiation of *CBP1* expression. Here, we demonstrated that the specificity of *CBP1* expression may be controlled by vegetative repression.

In this report, we also identified a negative regulatory region (NRS1) involved in the vegetative repression of *CBP1*. In NRS1, one interesting sequence, CCAAT, was detected. In *A. nidulans*, the CCAAT sequence and Hap complex regulate gene expression negatively (Steidl et al., 2001; Weidner et al., 2001); we focused on *MoHAPB* and the CCAAT sequence in NRS1 as one possible mechanism for the vegetative repression of *CBP1*. Derepression of *egfp* during vegetative growth was not induced by removal of the CCAAT sequence from NRS1. Moreover, in a *MoHAPB* disruptant, mRNA of *CBP1* was not detected in vegetative hyphae. Therefore, we concluded that the CCAAT sequence in NRS1 and *MoHAPB* did not have an important role in the vegetative repression of *CBP1*. Therefore, we are left with the question of what participates in *CBP1* repression. *CBP1* is involved in appressorium formation, and this process in *M. oryzae* is a specific differentiation step that cannot be found in model organisms such as *Saccharomyces cerevisiae* or saprophytic filamentous fungi, such as *Neurospora crassa* and *A. nidulans*. The elements involved in the negative regulation of *CBP1* may be specific to phytopathogenic fungi, and, therefore, remain to be identified.

Recently, in *M. oryzae*, important transcription factors involved in the differentiation step were reported. The zinc finger transcription factor *COS1* is involved in conidiophore stalk differentiation and conidiation (Zhou et al., 2009). The homeobox transcription factors *MoHOX2* and *MoHOX7* are involved in conidiation and appressorium formation, respectively (Kim et al., 2009). Interestingly, various conidiation-related genes were upregulated in a Δ *MoHOX2* mutant background. This fact suggests that the negative regulation of gene expression should be important for differentiation. The elements for the negative regulation of *CBP1* during vegetative growth may be also important for appressorium differentiation.

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