A Novel Gene, *CBP1*, Encoding a Putative Extracellular Chitin-Binding Protein, May Play an Important Role in the Hydrophobic Surface Sensing of *Magnaporthe grisea* During Appressorium Differentiation

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The conidial germ tube of the rice blast fungus, *Magnaporthe grisea*, differentiates a specialized cell, an appressorium, required for penetration into the host plant. Formation of the appressorium is also observed on artificial solid substrata such as polycarbonate. A novel emerging germ tube-specific gene, *CBP1* (chitin-binding protein), was found in a cDNA subtractive differential library. *CBP1* coded for a putative extracellular protein (signal peptide) with two similar chitin-binding domains at both ends of a central domain with homology to fungal chitin deacetylases and with a C-terminus domain rich in Ser/Thr related extracellular matrix protein such as agglutinin. The consensus sequence of the chitin-binding domain found in *CBP1* has never been reported in fungi and is similar to the chitin-binding motif in plant lectins and plant chitinases classes I and IV. *CBP1* was disrupted in order to identify its function. Null mutants of *CBP1* failed to differentiate appressoria normally on artificial surface but succeeded in normally differentiating appressoria on the plant leaf surface. Since the null mutant *Cbp1*- showed abnormal appressorium differentiation only on artificial surfaces and was sensitive to the chemical inducers, *CBP1* seemed to play an important role in the recognition of physical factors on solid surfaces.

Magnaporthe grisea (T.T. Hebert) Yaegashi & Udagawa (anamorph: *Pyricularia oryzae* Cava) is a heterothallic ascomycete and the causal agent of rice blast disease. Appressorium formation of *M. grisea* is considered a prerequisite process in order for this pathogen to penetrate host plants and cause the disease (Chumley and Valent 1990; Howard and Ferrari 1989; Howard and Valent 1996; Howard et al. 1991). Certain physical and chemical features of the host surfaces trigger appressorium formation in phytopathogenic fungi (Dean 1997; Mendgen et al. 1996; Tucker and Talbot 2001), and some of the molecular events triggered by these signals have been studied (Hamer and Talbot 1998).

Environmental factors such as the hydrophobicity of the contact surface (Lee and Dean 1994), surface hardness (Xiao et al. 1994a), light (Jelitto et al. 1994), plant cutin monomers (Gilbert et al. 1996), and nitrogen starvation (Talbot et al.

1997) have been proposed as cues for triggering appressorium differentiation by *M. grisea*. Most molecular biological approaches have focused on intracellular signal-transduction components such as cyclic AMP (cAMP) (Lee and Dean 1993a), G proteins (Liu and Dean 1997), and mitogen-activated protein (MAP) kinases (Xu and Hamer 1996) because of the extensive background information from other organisms. *M. grisea* is thought to accept at least a part of the environmental signals for appressorium differentiation through the heterotrimeric G proteins and then the signal is transferred to the second messenger, cAMP, through the adenylate cyclase. Downstream of the cAMP, it is thought that the signal is transferred into the nucleus and activates the appressorium formation-related genes through a kinase (cAMP-dependent protein kinase) cascade, MAP kinase cascade, or both. Although the whole pathway is not yet completely clarified, the importance of these signal-transduction components indicates that appressorium formation is a well-organized cellular differentiation. Such an intracellular signal-transduction pathway is occurring after sensing signals required for appressorium differentiation, but these sensing mechanisms are not well understood. To isolate new genes, traditional mutant analyses or insertional mutagenesis approaches using restriction enzyme-mediated integration (REMI) have been performed (DeZwaan et al. 1999; Sweigard et al. 1998).

Another strategy to isolate novel genes is differential cloning. A gene, *MPG1*, that is highly expressed during the infectious growth of *M. grisea* in its host was identified by differential screening (Talbot et al. 1993). *MPG1*, which encodes a hydrophobin, is expressed during conidiation and in a mycelial culture starved for nutrients. This gene appears to be involved in appressorium formation on the hydrophobic surfaces (Beckerman and Ebbola 1996; Talbot et al. 1993, 1996). Genes highly expressed during appressorium formation were also collected by Lee and Dean (1993b). However, their functions have not yet been reported.

We previously reported that a cDNA library that contained sequences expressed during the early stage of appressorium differentiation was constructed by a cDNA subtraction strategy with appressorium-differentiating germ tubes and vegetative-growing mycelia (Kamakura et al. 1999). The number of distinct clones in the library was estimated to be approximately 200, and thus, functional analysis of those genes seemed to be practical. In this paper, we report the finding that one unique

gene from this collection may play an important role in the recognition of physical factors that exist on artificial solid surface and trigger the differentiation of appressorium.

RESULTS

Gene structure of *CBP1*.

As previously reported (Kamakura et al. 1999), the gene *CBP1* (formerly called clone A4) was specifically expressed in the germling and was repressed completely (undetectable even by reverse-transcription-polymerase chain reaction [RT-PCR]) in vegetative mycelium. We could not clearly detect the expression of *CBP1* in spores or appressoria simply because we have not succeeded in preparing high-quality RNA samples from those cells of the P2 strain. A 7,406-base pair (bp) region of genomic sequence (DDBJ accession no. AB064264) containing *CBP1* was determined from a cosmid clone, 26-7. Compared with a cDNA clone (2,531 bp without polyA, probably complete length), *CBP1* appeared to have seven introns (Fig. 1). The fifth intron was 795 bp long, which was atypically longer than most fungal introns. The seventh intron was located downstream of the stop codon (in 3' untranslated region). Experimentally determined intron signal sequences are shown in Table 1. From the results, *M. grisea* seems to have a more relaxed sequence requirement for intron signal sequences than does *Neurospora crassa*. The molecular weight of the *CBP1* product (Cbp1p) calculated from the expected open reading frame (ORF) (590 amino acids [aa]) was confirmed by expression of the Cbp1p in *Escherichia coli* (K.-i. Saitoh, unpublished data).

Although no significantly homologous gene was found by BLAST searches (blastn, blastx, and tblastx with full *CBP1* sequence; and blastp and tblastn with full Cbp1p sequence), internal domains exhibited significant homology to a chitin-binding domain (chitin-recognition or -binding motif signature; Prosite-ID: PS00026 C-x(4,5)-C-C-S-x(2)-G-x-C-G-x(4)-[FYW]-C) by using blastp and tblastn with the Cbp1p sequence. Two chitin-binding domains were located in the Cbp1p as shown in Figure 1. The second motif lacked the first cysteine residue consensus in the chitin-binding motif signature. Interestingly, this domain was homologous to the chitin-binding domain of plant chitinases classes I and IV (family 19 chitinase; Henrissat

and Bairoch 1996) and to chitin-binding plant lectins such as wheat germ agglutinin (WGA). Although WGA has four similar chitin-binding domains, plant chitinases have only one chitin-binding domain (Collinge et al. 1993). Recently, a fungal chitin deacetylase with this type of chitin-binding domain appeared in the database (*CHD* gene of *Blumeria graminis*, GenBank accession no. AY039008). These putative gene products (Chdp of *CHD* and Cbp1p of *CBP1*) are the first to be reported on a protein with this type of chitin-binding domain in organisms other than plants. The Cbp1p amino acid sequence located between the two chitin-binding domains showed homology to fungal and bacterial chitin deacetylase or bacterial *nodB* genes, which are also known as *N*-deacetylase (Stacey 1995). The most homologous sequence was that of the Chdp, mentioned above (blast score = 100 bits, expect = 1e-24). Almost the entire region of the Chdp (324 aa) was homologous (43% of the amino acids were identical) to the part of the Cbp1p from its first chitin-binding domain (Fig. 1). None of other genes had same kind of chitin-binding domains as did the Chdp, and the Chdp terminated before the latter half of the Cbp1p sequence, including the second chitin-binding domain. *CHD* had five introns, but there was no coincidence in the position of introns with *CBP1*. After its second chitin-binding domain, the Cbp1p has a domain rich in serine and threonine (456 to 534 aa). Among these 79 aa, 44 (55.7%) were serine and 17 (21.5%) were threonine (total 77.2%). Some yeast extracellular chitinases contain a region rich in serine and threonine (Kuranda and Robbins 1991). A short (20 to 30 aa) serine/threonine-rich domain was observed in the chitin deacetylase of *Mucor rouxii* (Kafetzopoulos et al. 1993). However, we could not find fungal chitin-related enzymes (chitin synthase, chitinase, and chitin deacetylase) containing an intensive and long serine/threonine-rich domain as the one in the Cbp1p. At the N terminal of *CBP1*, a 17-aa sequence that could function as a secretory signal peptide was found by using signalP, V1.1 program (Nielsen et al. 1999). Except for this signal peptide region, the Cbp1p is a hydrophilic protein and no apparent transmembrane domain was found.

Targeted disruption of the *CBP1* gene.

A vector for targeted gene disruption was constructed as shown in Figure 2A. *M. grisea* strain P2 was transformed with

Fig. 1. Structure of *CBP1*. Boxes in the *CBP1* genomic structure are exons (Ex). Zigzag lines between exons indicate introns. Features in the Cbp1 product are placed at the corresponding position of cDNA of *CBP1*. Chdp = product of *CHD* (chitin deacetylase) of *Blumeria graminis*.

this vector. Out of 130 blasticidin S resistant transformants, 32 colonies were analyzed by PCR (data not shown) with *CBP1*-specific primers. Among the candidate colonies that gave no amplification with these primers, two independent disruptants were confirmed by Southern hybridization (Fig. 2B). Since the disruption vector has only the flanking sequence outside the ORF of *CBP1*, these disruptants were null mutants. The two disruptants, *CBP1*-D9 and *CBP1*-D25; one ectopic transformant *CBP1*-D2; and the parent strain P2 were analyzed phenotypically. In all experiments, *CBP1*-D9 and *CBP1*-D25 were indistinguishable. *CBP1*-D2 showed indistinguishable phenotypes from parent strain P2. Thus, the null mutants *CBP1*-D9 and -D25 are renamed as *Cbp1*-.

Phenotypes of the *Cbp1*- disruptants.

Appressorium differentiation of the *Cbp1*- disruptants was assayed on polycarbonate surface (described below). As shown in Figure 3A and B, strain P2 and ectopic transformant *CBP1*-D2 differentiated appressorium efficiently (approximately 90%) 8 hr after inoculation. However, the ability of *Cbp1*- to differentiate appressorium was dramatically reduced (less than 10%) and long germ tubes were observed on polycarbonate at 8 h (Fig. 3C). No other defects of mycelium growth, conidiation, or pigmentation were detected (data not shown). Much of the appressorium formation ($17.0\% \pm 9.8\%$) by *Cbp1*- was observed near the edge of the water drop (Fig. 3D), and we observed fewer appressorium-forming germ tubes in other parts of the droplet ($4.6\% \pm 4.0\%$). The reason for this phenomenon is unknown. The same assay was performed on other artificial solid surfaces, such as Teflon (DuPont, Wilmington, DE, U.S.A.), cellophane, the hydrophobic surface of GelBond film (FMC BioProducts, Rockland, ME, U.S.A.), hydrophobic TiO_2 , and Mylar (DuPont). On every surface tested, *Cbp1*- was unable to differentiate appressoria efficiently, as observed on the polycarbonate surface, and no significant difference was observed among these surfaces (data not shown). No appressorium formation was observed on the hydrophilic surface of GelBond film and hydrophilic TiO_2 , as it was for strain P2. After a longer incubation (24 h), the tip of the germ tube did swell to some extent (5 to 30%), even in the middle of the water drop (Fig. 3E and F). At 48 h, development slowly progressed and 20 to 50% of the germ tubes formed appressoriumlike swellings and slight melanization was observed.

The effect of cAMP was tested to determine whether the *Cbp1*- mutant defect is in the earlier point of the intracellular signal-transduction pathway carried by cAMP. Since we have observed that 3-isobutyl-1-methylxanthine (IBMX) and 8-bromo cAMP were more effective than cAMP on our parental strain P2 (S. Yamaguchi, *unpublished data*), we used IBMX for the test. IBMX is an inhibitor of cyclic nucleotide phosphodiesterase that down regulates the concentration of cellular cAMP. The inhibition of the enzyme causes the accumulation of intracellular cAMP, giving an effect similar to that of adding cAMP from outside. The phenotype of *Cbp1*- was retrieved by

2.5 mM IBMX (Fig. 3G), which means *CBP1* works in the early stage of the signaling cascade of appressorium differentiation. As another second messenger of signal-transduction pathway, diacylglycerol (DAG) is known to be effective in the formation of appressorium (Thines et al. 1997). DAG could also rescue the defective appressorium formation phenotype of *Cbp1*-, as did IBMX (data not shown).

Interestingly, *Cbp1*- disruptants were as pathogenic on rice as was the parent strain P2 (Fig. 4A). Appressorium formation was observed on the rice sheaths and leaves without a detectable delay (K.-i. Saitoh, *unpublished data*). To determine the ability of *Cbp1*- to recognize the chemical signals that may exist on the plant surface, 1,16-hexadecanediol (hereafter referred to as hexadecanediol), which is reported as a chemical inducer of appressorium formation (Gilbert et al. 1996), was applied. In the report, it was observed that less than 1 μM hexadecanediol was needed to induce appressorium formation. However, our strain P2 was a little less sensitive to hexadecanediol, and 1% ethanol (experimental final concentration of the solvent for hexadecanediol) had a weak inductive effect on *Cbp1*-mutants, so we added 10 μM hexadecanediol to analyze clearly. The fatty alcohol induced the appressorium formation of *Cbp1*- on polycarbonate (Fig. 4C). The actual chemical material released from the rice surface to induce the appressorium formation of *Cbp1*- has not been determined. We have also tried to rescue the mutant defect by adding hexadecanediol or IBMX onto the GelBond hydrophilic surface. Both hexadec-

Fig. 2. Targeted gene disruption of *CBP1*. **A**, The physical map around the *CBP1* genomic gene and predicted *Cbp1*- null mutant obtained by homologous recombination with the gene-disruption vector. *BSD* indicates the blasticidin S resistance marker cassette that consists of the open reading frame of the *BSD* (blasticidin S deaminase) gene and *trpC* promoter and terminator. Restriction sites: E = *EcoRI*, H = *HindIII*, N = *NdeI*, P = *PstI*, and S = *SacI*. **B**, Southern analyses of gene disruptant. Samples: M, molecular weight marker lane (λ *HindIII* [digoxigenin labeled]); lanes 1 and 4, ectopic transformant *CBP1*-D2; lanes 2 and 5, gene disruptant *CBP1*-D9; and lanes 3 and 6, host strain P2. Lanes 1 to 3, *HindIII* digest of genomic DNA. Lanes 4 to 6, *SacI* digest of genomic DNA. Lane 7, *PstI*-digested gene-disruption construct (one *PstI* site exists inside the *BSD* cassette).

Table 1. Intron signal sequences in *CBP1*

Introns	Acceptor site ^a	3' Splice sequence
1	ACTAAC	CAG
2	GCTGAC	CAG
3	ACTGAC	CAG
4	ACTAAT*	CAG
5	T*CTGAC	CAG
6	ACTC*AC	CAG
7	GA*TAAC	A*AG
Consensus in <i>Neurospora crassa</i>	RCTRAC	YAG

^a * = Disagreement with consensus sequence.

anediol and IBMX could restore the ability of Cbp1- to form appressorium to some extent (at 6 h, 35.0% $\pm$ 9.9% by hexadecanediol and 50.1% $\pm$ 17.9% by IBMX) on the hydrophilic surface. However, both chemicals were more effective in inducing appressorium formation of Cbp1- on polycarbonate (60 to 80% appressorium formation at 6 h) and of parental strain P2 on the hydrophilic surface (approximately 80% appressorium formation).

Since the chemical-sensing pathway, intracellular signal-transduction pathway, and ability of morphological and functional development including pathogenicity seem to be functional in Cbp1-, it suggests that Cbp1- is a mutant that fails to recognize the inductive cue or cues for appressorium differentiation existing on artificial surfaces.

Complementation of the *CBP1* gene.

Because the parent strain P2 is a female-sterile strain and male fertility is extremely low (S. Yamaguchi, *unpublished data*), genetic analysis to confirm the linkage of the *CBP1*-null genotype and the Cbp1- phenotype is impossible. Although we have isolated two independent disruptants and both of them showed indistinguishable phenotypes, as described above, more conclusive experiments are required. To confirm the linkage of genotype and phenotype, we transformed the Cbp1-

with the full-length cDNA of *CBP1* under the control of a *trpC* promoter (Fig. 5A). Five independently isolated transformants were analyzed for appressorium formation on polycarbonate. All of them differentiated appressoria to 50 to 80% of the germ tubes in 6 h (Fig. 5B), showing that the appressorium-deficient phenotype of Cbp1- was caused by the inactivation of the *CBP1* gene.

DISCUSSION

Appressorium differentiation in *M. grisea* involves the sensing of complex environmental cues such as surface hydrophobicity, surface hardness, chemicals, and light. The relative importance of these signals in the induction of appressorium differentiation is not known. However, a physical factor is considered to be sufficient for appressorium formation, because *M. grisea* efficiently forms appressoria on various artificial solid surfaces with no common chemical components. On the other hand, some chemical inducers such as hexadecanediol can induce appressorium on noninductive surfaces such as glass (Gilbert et al. 1996). From these phenomena, it has been speculated that either a physical factor or a chemical factor can independently trigger appressorium differentiation. The phenotype of the null mutant of an α -subunit of heterotrimeric G protein (MagB-) suggested the independency of physical and chemical induction of appressorium formation (Liu and Dean 1997). However, MagB- mutants had not only a defect in appressorium formation, but also in a number of cellular responses, such as conidiation, sexual development, and mycelial growth. Compared with the MagB-, the phenotype of Cbp1- was strictly limited to the induction of appressorium formation on artificial solid surfaces.

Other than *MAGB*, there are many appressorium-related genes cloned or genetically identified, such as *MPG1* (Talbot et al. 1993); *CPKA* (Mitchell and Dean 1995); *PMK1* (Xu and Hamer 1996); *MAC1* (Choi and Dean 1997); *PTH11* (DeZwaan et al. 1999); *CON1* and *CON7* (Shi and Leung 1995); *APF1* (Silu et al. 1998); *APP1*, *APP2*, and *APP3* (Zhu et al. 1996); and *APP5* (Chun and Lee 1999). *MPG1*, *CPKA*, *PMK1*, *MAC1*, and *PTH11* are already cloned and characterized. Among the noncloned genes, *CON1*, *CON7*, and *APF1* are apparently different from *CBP1*, because their mutants show reduced or no pathogenicity to rice and a morphological change in the conidia. The mutants *app1* and *app5* also showed defective pathogenicity and they were not rescued by cAMP. Conidiation of *app1*, *app2*, and *app3* mutants was reduced compared with that of the wild type. Although all mutants mentioned here show at least one difference with Cbp1-, the *app2* mutant seems to have a phenotype quite similar to Cbp1-. In spite of the appressorium-deficient phenotype (less than 10% differentiation on the artificial hydrophobic surface), the *app2* mutants are fully pathogenic (Dean et al. 1994) and its germ tubes are long and smooth without making a "hook" on the artificial hydrophobic surface. There are two different phenotypes between *app2* and Cbp1-. First, conidiation of *app2* was reduced significantly (less than 25%) compared with that of the wild type. Cbp1- did not show a detectable reduction on conidial formation. Second, *app2* was not rescued by cAMP on the hydrophilic surface, but Cbp1- could form appressoria by the addition of IBMX on the hydrophilic surface (efficiency of appressorium formation was lower than that of wild type). It is possible that the effects of cAMP and IBMX are somehow different. We could not clearly answer this question, because our strain P2 was less sensitive to cAMP, even on the hydrophobic surface. Including this phenomenon, differences of the genetic background between the strains might affect the slight difference of phenotypes. Considering that, we cannot at this moment judge that *APP2* and *CBP1* are different genes. Cloning of *APP2* or a

Fig. 3. Appressoria formation assay on polycarbonate. **A**, Parent strain P2 8 h after inoculation. **B**, Ectopic transformant CBP1-D2 (8 h after inoculation). **C**, Cbp1- null disruptant CBP1-D9 (8 h after inoculation). Differential interference microscopic image. **D**, Appressorium formation in the edge of water drops (6 h after inoculation). Arrow indicates the edge of the water drop. **E** and **F**, Formation of appressoriumlike swelling by Cbp1- after a long incubation (24 h). **G**, Appressorium formation of Cbp1- by 2.5 mM 3-isobutyl-1-methylxanthine (IBMX) (8 h after inoculation). **A** to **G**, Bars = 20 μ m. **A**, **B**, and **D** to **G**, Bright-field microscopic images.

complementation experiment of *app2* by *CBP1* is awaited to answer this question.

After sensing one of the sufficient environmental factors, *M. grisea* should activate intracellular signal-transduction pathways to differentiate appressorium. Gene-disruption experiments on the adenylate cyclase (*MAC1*; Choi and Dean 1997), cAMP-dependent protein kinase (*CPKA*; Mitchell and Dean 1995; Xu et al. 1997), and MAP kinase (*PMK1*; Xu and Hamer 1996) strongly support the involvement of these signaling pathways. Germ tubes of *Cbp1*- were long and smooth, but sometimes form a "hook" (Fig. 4B), which is considered to be one of the first visible symptoms for appressorium formation. Formation of a long, smooth germ tube is similar to that of the null mutant of the cAMP-dependent protein kinase (*CpkA*-; Mitchell and Dean 1995), *app1*, *app2*, and *MagB*-. Although the efficiency is different (>90% with *CpkA*- and 5 to 30% with *Cbp1*-), *CpkA*- mutants formed appressoria after a long incubation (24 h) (Xu et al. 1997), as did *Cbp1*-. However, *Cbp1*- was apparently different from the *CpkA*- phenotype, since it is fully pathogenic.

Compared with the signal-transduction pathway, sensing mechanisms triggering appressorium differentiation are still unknown. *PTH11* may function as a sensor at the early stage of appressorium differentiation (DeZwaan et al. 1999). The *Pth11p* seemed to be an upstream effector of appressorium differentiation in response to cues existing on the contact surfaces, because the ability of *Pth11*- to form appressorium was rescued by cAMP and DAG. However, *Pth11*- has a reduced ability to differentiate appressoria, even in the presence of hexadecanediol, which could mean that *PTH11* has some effect on the sensing pathway of this chemical inducer or that there might be some cross talk between contact surface sensing and the hexadecanediol signal pathway. It was reported that hexadecanediol was ineffective in the formation of appressorium if germ tubes were not attached on solid surfaces (Gilbert et al. 1996).

Another gene that may play an important role in contact surface sensing is *MPG1* (Talbot et al. 1993). This gene is highly expressed in the germ tubes of *M. grisea*. *MPG1* encodes hydrophobin, which forms a rodlet layer on the surface of the cell wall to give the hydrophobic character to the cell surface. Disruptants of *MPG1* (*Mpg1*-) form appressoria inefficiently (less than 10% than that of wild type) and show reduced pathogenicity. Since *Mpg1*- was rescued by cAMP, the *Mpg1p* could work on the early stage of surface recognition. Interestingly, *Mpg1*- reduced its appressorium formation ability drastically on highly hydrophobic surfaces such as Teflon or rice leaves but retained the ability on less hydrophobic surfaces, such as the hydrophobic surface of GelBond and glass surfaces (Beckerman and Ebbole 1996). They also found that the *Mpg1p* acts in trans by assaying the appressorium formation with a mixture of a null mutant and wild-type strain. Interestingly, both the *Mpg1p* and *Cbp1p* have repeated Cx(n)CCx(n)C motifs. In the *Cbp1p*, two of these motifs are chitin-binding domains (Cx(4)CCSx(4)C and x(4)CCSx(4)C). We also found the third Cx(n)CCx(n)C motif after the Ser/Thr cluster. The relationship of these three motifs and a similar motif in hydrophobin is unknown. However, since hydrophobin is a self-assembly protein, the third motif in the *Cbp1p* may have some interactive ability to other *Cbp1p* or unknown proteins. Compared with the *Mpg1*-, *Cbp1*- showed an indistinguishable phenotype on various artificial surfaces, from Teflon (highly hydrophobic) to cellophane (cellulose membrane, relatively hydrophilic). We have examined the ability of the wild-type *Cbp1p* to complement the null mutant phenotype in trans, as did the *Mpg1p*, and observed that the *Cbp1p* could not act in trans (data not shown).

The importance of surface hydrophobicity for appressorium formation is somehow confusing. It is apparent that *M. grisea*

forms appressorium on highly hydrophobic surfaces but not on highly hydrophilic surfaces, such as the hydrophilic surface of GelBond. However, there are other observations that indicate that surface hardness is more important than hydrophobicity (Xiao et al. 1994a) or that hydrophobicity is not a clear inducing factor of appressorium differentiation (Jelitto et al. 1994). It is difficult to explain why *Mpg1*- form appressorium on relatively hydrophilic surfaces, even if it shows a defective phenotype on host surfaces. If at least some parts of the *Mpg1p* could work as a hydrophobicity conditioner of contact surfaces, it might be possible for the *Mpg1p* to control the hydrophobicity of highly hydrophobic surfaces to a suitable level for appressorium formation, while no evidence for such function of the *Mpg1p* has yet been revealed. *Pth11*- also showed different sensitivity on various hydrophobicity (DeZwaan et al. 1999), although this phenotype varied strain to strain. Compared with that of the *Pth11*-, the phenotype of *Cbp1*- is more uniform on surfaces with various hydrophobicity. The *Cbp1p* may be involved in a sensing mechanism for other common physical factors existing on the various kinds of solid surfaces.

The appressorium formation of *Cbp1*- at the edge of a water drop is an interesting phenomenon. It is difficult to explain this phenomenon, because few differences between the edge and center of a water drop could be expected. One of the possible differences is the concentration of oxygen. The surface tension could also be different between the edge and center of a water drop. In the latter case, it seems to be related somehow to the strength of cellular attachment to the solid surface. We have tried to add a very low concentration of detergent in order to reduce the surface tension. The addition of 0.00025% Tween 80 affected the shape of a water drop, but no remarkable difference was observed on the appressorium formation in the edge of the water drop. At a higher concentration, conidia were not dispersed evenly in the water drop. To date, we have not been able to design other conclusive experiments to reveal the reason of this phenomenon.

From the comparison of *Cbp1*- and other appressorium-defective mutants, we infer that the phenotype of *Cbp1*- is the simplest and clearest evidence reported so far to prove the independency and sufficiency of physical and chemical inducers for the appressorium formation of *M. grisea*. *Cbp1*- easily retrieved its ability to form appressorium by an unknown factor from the rice leaf, cAMP (IBMX), hexadecanediol, and even by an unknown factor generated in the edge of the water drop.

Fig. 4. Pathogenicity and effect of the chemical inducer, 1,16-hexadecanediol. **A**, Pathogenicity assay by spray of conidia. Photographs were taken 8 days after inoculation. **B**, *Cbp1*- on polycarbonate in a water drop. Photographs were taken 4 h after inoculation. Arrow indicates rarely observed hooking event. **C**, Induced appressorium development of *Cbp1*- by 10 μ M 1,16-hexadecanediol (4 h after inoculation).

These properties suggest that the Cbp1p is one of the most upstream components of the surface-sensing system of *M. grisea*. It may be possible to search the sensing system for chemical inducers by using the Cbp1- mutant as a host strain.

Because the firm attachment of spore and germ tube on the contact surface is one of the most important factors to induce appressorium differentiation (Xiao et al. 1994b), the Cbp1p may be a component of adhesive materials for germ tube attachment. However, no drastic reduction of cellular attachment was observed by our preliminary experiment with a sonicator (Ohtake et al. 1999) (data not shown). Although it is still possible that the Cbp1p is one of the minor components of the adhesive materials and that the loss of the Cbp1p causes some slight reduction of cellular attachment, the change of the phenotype of Cbp1- was so drastic compared with the difference of the efficiency of appressorium formation on various solid materials, each of which probably had various surface affinity to the adhesive materials (mucilage) around the germ tube.

If the Cbp1p is not a component of cellular attachment, what kind of function can we expect? The biochemical properties of the Cbp1p are not yet revealed, because the Cbp1p was extremely hard to detect using the commercially available *in vivo* and *in vitro* translation systems we have tried so far. We could only confirm the expected molecular weight of the Cbp1p by a thin signal of western hybridization with the antibody against His₆-tag of the Cbp1p fusion protein. However, in the deduced amino acid sequence and gene structure of *CBP1*, there are many interesting features. The former half of the ORF (in exons 1 to 5) has homology with chitin deacetylase with two chitin-binding domains on both ends and a presumable signal peptide at its N terminus. Especially, this former half contains a ho-

mologous sequence with an almost whole ORF of the chitin deacetylase gene *CHD* of *Blumeria graminis*. Since we could not find structural biological studies on chitin deacetylase, we could not determine whether this homologous region could work as a chitin deacetylase. The latter half (in exons 6 and 7) starts with a long Ser/Thr cluster that has not been found in chitin deacetylases so far. A shorter Ser/Thr cluster was observed in some fungal extracellular chitin deacetylases (Kafetzopoulos et al. 1993). We are now trying to determine the chitin-binding ability of the Cbp1p and its chitin deacetylase activity. Interestingly, the chitin-binding domains of the Cbp1p and Chdp were first found in fungal proteins such as chitinase, chitin deacetylase, and chitin synthase. The highest similarity was found among the class I and class IV chitinase and chitin-binding lectins of plants, including the rice plant. Even among the plant chitinases classes I and IV, the dual chitin-binding domains have not yet been reported and the existence of multiple chitin-binding domains is a property of lectins. The Chdp also contains only one chitin-binding domain. This sequence motif of the chitin-binding domain is considered to be capable of binding *N*-acetylglucosamine if proper conformation was formed (Wright 1992). In addition, the Ser/Thr cluster was similar to the cell wall protein or extracellular matrix glycoproteins, such as agglutinin.

Taking everything into account, we infer that the Cbp1p is a lectinlike extracellular chitin-binding protein containing a homologous region with chitin deacetylase. The Cbp1p is probably involved in the sensing of factors from solid surfaces that induce appressorium differentiation. It is difficult to explain how these structural characters contribute to surface sensing. Existence of chitin-binding domains suggests that the Cbp1p could be localized to the cell wall. If the Ser/Thr cluster expands outside the cell wall, the Cbp1p may act as a component of cellular attachment or signal carrier from the outside of the cell wall. If the Ser/Thr cluster expands inside the cell wall, the Cbp1p may play a role in signal triggering, like extracellular matrix sensor proteins, by means of interaction with membrane protein or proteins. More analyses are in progress. Those structural features of *CBP1* also seem to be interesting from the evolutionary point of view. Recently, we have noticed that in the *Neurospora* Database there is at least one possible sequence to be translated into the same type of chitin-binding domain of the Cbp1p. We are planning to confirm whether the sequence really codes an expressed gene and to search genes closely related to the each component of *CBP1* from other phytopathogenic and nonphytopathogenic fungi and plants.

MATERIALS AND METHODS

Fungal strains and culture conditions.

M. grisea strain P2, a Japanese rice-pathogenic isolate, is a stock culture in this laboratory and used throughout this study. Cultures were maintained on potato dextrose agar (Difco, Sparks, MD, U.S.A.) at 25°C. For conidiation, the fungus was grown on oatmeal agar medium (Difco) at 27°C for 2 weeks. Conidiation was induced by placing the plates under BLB lamps (FL20S, 20 W; Toshiba Co., Ltd., Tokyo, Japan) after removing the aerial mycelia with a sterilized brush. After 3 days of incubation under BLB lamps, conidia formed were brushed off into distilled water and then collected by centrifugation.

Construction of full-length cDNA and cosmid library.

RNA preparation from a germ tube was the same as described in a previous report (Kamakura et al. 1999). A full-length cDNA library was constructed using the CapFinder PCR cDNA Synthesis Kit (Clontech, Palo Alto, CA, U.S.A.). Synthesized cDNAs were size-separated by SizeSep 400 SpunColumn

Fig. 5. Complementation of Cbp1- by full-length cDNA of *CBP1*. **A**, Full-length cDNA was introduced between the promoter and terminator of *trpC* (*PtpC*: promoter of *trpC*; *TtrpC*: terminator of *trpC*). The resulted plasmid pKS01 was introduced into the Cbp1- disruptant with pCSN43 and selected as hygromycin B resistance transformants. **B**, Phenotype of the typical cotransformants, which were confirmed to retain the cDNA sequence of *CBP1* and mRNA is expressed. Picture was taken 6 h after inoculation. Hygromycin resistance transformants lacking the *CBP1* sequence showed indistinguishable phenotype with the Cbp1- disruptant (data not shown).

(Amersham Pharmacia Biotech, Piscataway, NJ, U.S.A.) to remove the molecules shorter than 400 bp, and elute was introduced into the pCR2.1 vector (Invitrogen, Carlsbad, CA, U.S.A.) to construct the *E. coli* library.

A cosmid library of the genome of *M. grisea* strain P2 was constructed using the SuperCos1 Cosmid Vector Kit (Stratagene, La Jolla, CA, U.S.A.). Approximately three genome equivalent cosmid libraries (about 3000 colonies) were maintained as a grid library.

General techniques such as DNA manipulation and Southern analysis were performed following the "Molecular Cloning" (Sambrook et al. 1989).

DNA sequencing was determined with the ALF-Express DNA sequencer (Amersham Pharmacia Biotech). Sequence data was analyzed by GeneWorks ver. 2.5.1 software (IntelliGenetics, Mountain View, CA, U.S.A.). Database searches were performed with the BLAST programs (Altschul et al. 1990).

Plasmid vectors for targeted gene disruption and complementation.

A local restriction map around *CBP1* was made as shown in Figure 2A by using the sequence data and Southern hybridization analysis and cosmid mapping (data not shown) in order to design the gene-disruption construct. pBF101MBD, a modified vector of pBF101 (Kimura et al. 1994), harboring the *BSD* (blastidicin S deaminase) gene driven by the *trpC* promoter and terminator (Mullaney et al. 1985) as a selectable marker was used for the backbone of the gene-disruption constructs. In pBF101MBD, most of the restriction sites inside the *trpC-BSD* expression cassette of pBF101 were removed or changed to indigestible, except the *Clal*, *PstI*, *SmaI*, and *MluI* sites. The other part of pBF101MBD, including multiple cloning sites adjacent to the *BSD* expression cassette, was the same as pBF101. The 5' and 3' flanking regions of *CBP1* were introduced into the *SalI* and *XbaI* sites of pBF101MBD adjacent to the *BSD* expression cassette. Original pBF101 was used for the complementation experiments. The *BSD* gene was removed by *EcoRI* digestion and replaced by the full-length cDNA of *CBP1* to be expressed under the *trpC* promoter. Since the gene disruptant cell is already blastidicin S resistant, pCSN43 (Staben et al. 1989) harboring *hph* (hygromycin phosphotransferase gene) was cotransformed to select the transformants by hygromycin B resistance.

Transformation of *M. grisea* and extraction of genomic DNA.

Protoplasting and transformation of *M. grisea* with selectable marker *BSD* or *hph* were performed as described before (Kimura et al. 1995). Blastidicin S was purchased from the Funakoshi Pharmaceutical Co. (Tokyo, Japan). Extraction of genomic DNA from fungal mycelia was performed as described below. Protoplasts were incubated in 5 ml of extraction buffer (0.1 M EDTA; 10 mM Tris-HCl, pH 8.0; 0.5% sodium dodecyl sulfate) in a 15-ml tube for 10 min at 56°C followed the addition of 100 µg of Proteinase K per ml (Roche Diagnostics, Tokyo, Japan) and 60 min of incubation at 56°C. Cellular extract was then subjected to phenol extraction and ethanol precipitation, and finally, precipitated DNA was recovered from the solution by a glass rod and air-dried after a rinse with 70% ethanol.

Appressorium formation and pathogenicity assay.

Polycarbonate is a hard, moderately hydrophobic and highly appressorium inductive substrate (Xiao et al. 1994a). Polycarbonate plates were used for the appressorium formation assay. Polycarbonate sheets (1 mm thick) were purchased from Sanplatec Corp. (Osaka, Japan). A sheet of polycarbonate was cut with scissors into plates the same size as slide glasses, and the

polycarbonate plates were then washed by shaking in distilled water and dried completely before use. Conidial suspensions (15 to 20 µl; 3 to 5 × 10⁴ conidia per ml) were dropped on the polycarbonate plates and incubated for an appropriate period in square plastic boxes with a damp paper towel to maintain moisture. Appressorium formation was observed and counted under bright-field microscopy at 6, 12, and 24 h after inoculation. Conidial suspensions containing 2.5 mM IBMX (Sigma-Aldrich, St. Louis, MO, U.S.A.) or 10 µM 1,16-hexadecanediol (Nakarai Tesque, Kyoto, Japan) were used to assay the effect of chemical inducers on appressorium formation.

Rice (*Oryza sativa*) cultivar Koshihikari, which is susceptible to strain P2, was used for the pathogenicity assay. Plants were cultivated for 2 weeks at 26°C and then inoculated with a conidial suspension (1 × 10⁶ conidia per ml, 1 ml for each plant) by spray using an airbrush. After spraying, seedlings were placed in a humid chamber overnight and then incubated at 26°C. Symptoms were recorded at 8 days after inoculation.

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