Overexpression of a Gene Encoding a Catabolite Repression Element in *Alternaria citri* Causes Severe Symptoms of Black Rot in Citrus Fruit

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**ABSTRACT**


A gene (AcCreA) encoding a catabolite repression element (CreA) with two zinc fingers of the Cys2His2 type was isolated from the postharvest fungal pathogen *Alternaria citri*. The AcCreA overexpression mutant AcOEC2 of *A. citri* showed normal growth on pectin medium and on segments of peel or the juice sac area from citrus fruit. Production of endopolygalacturonase, an essential virulence factor of this pathogen, was similar in AcOEC2 and the wild type in pectin-containing media. However, addition of glucose to the medium showed that carbon catabolite repression of endopolygalacturonase gene (*Acpg1*) expression, as well as endopolygalacturonase production, was lost in AcOEC2. The wild-type strain of *A. citri* causes rot mainly in the central axis of citrus fruit without development of rottig in the juice sac area; however, AcOEC2 caused severe black rot symptoms in both the central axis and juice sac areas. These results indicate that AcCreA-mediated catabolite repression controls the virulence or infection of this pathogen, and that the wild-type *A. citri* does not cause symptoms in the juice sac area due to carbon catabolite repression by sugars in the juice of the juice sac area.

Additional keywords: cell wall-degrading enzyme.

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*Alternaria citri* Ellis & N. Pierce in N. Pierce causes the postharvest disease *Alternaria* black rot of a broad range of citrus fruit (4,5). *A. citri* infects the stem end of the fruit and causes internal black discoloration and maceration of the fruit core around the central axis area during storage (4,5).

Fungal pathogens, including *A. citri*, produce extracellular enzymes that can degrade plant cell wall polymers during infection, and various aspects of their potential roles in pathogenicity have been examined, including penetration, maceration, nutrient acquisition, plant defense induction, and symptom expression (7,8,42). The involvement of CreA in glucose repression has been examined in several fungi (30,32,44); however, the effect of carbon catabolite repression and, hence, CreA on the pathogenesis of phytopathogenic fungi is still not clear.

In this study, we examined the role of CreA in regulation of the expression of the endoPG gene, *Acpg1*, which is the established essential dominant virulence factor in *Alternaria citri* (17,18), because analysis of the *Acpg1* promoter region revealed the presence of putative binding sites for CreA (26). We cloned a creA gene, designated *AcCreA*, from *A. citri* and made an overexpression mutant with *AcCreA*. Phenotypic examinations of the overexpression mutant showed an unexpected loss of carbon catabolite repression, and the mutant gained the ability to cause rotting in the juice sac area of citrus fruit.

**MATERIALS AND METHODS**

Fungal culture conditions and measurement of endoPG activity. Mycelial fragments of *A. citri* (American Type Culture....
Collection 58171) (9) and the CreA overexpression mutant AcOEC2 were stored in 25% (vol/vol) glycerol at –80°C and were grown on V8 agar plates immediately before use in various experiments. They were grown in potato dextrose broths (PDB) or in liquid pectin or pectin agar medium containing 1% (wt/vol) pectin (P-9135; Sigma-Aldrich, St. Louis) with or without addition of 2% (wt/vol) glucose, as described previously (16–18). The fungi were grown in 200-ml flasks each containing 50 ml of pectin liquid medium for 10 or 25 days at 24°C, the culture filtrates were filtered through four layers of gauze for endoPG enzyme assays, and the mycelia mats were recovered for isolation of nucleic acids. EndoPG activity of the cultures was measured by a relative viscosity assay, and one unit was defined as the activity that reduces the viscosity of a 1.5% polygalacturonic acid solution (pH 5.0) at 37°C in 5 min to 50%, as described previously (16).

**Isolation of the A. citri gene encoding CreA.** Genomic DNA from wild-type A. citri was isolated from the mycelial mats grown in PDB by the method of Isshiki et al. (17). Total RNA was isolated from mycelial mats grown at 24°C for 14 days in PDB or for 10 or 25 days in pectin liquid medium, according to the method of Masunaka et al. (23).

A pair of polymerase chain reaction (PCR) primers, CREAF5 (5′-GARAARCNCAYGCGNTG-3′, forward primer) and CREAR5 (5′-GTRTGRTCNGGNGTNGG-3′, reverse primer) (Table 1), designed from the conserved region of deduced amino acid sequences of CreA from several fungal phytopathogens, was used for PCR amplification of a portion of the A. citri gene encoding CreA. PCR was performed in a Gene Amp PCR System 9700 (Applied Biosystems, Foster City, CA) at 95°C for 1 min, followed by 30 cycles of 2 min at 95°C, 2 min at 50°C, and 1 min at 72°C, and a final extension of 10 min at 72°C. The PCR reactions were run in a 10-µl volume, and 2 µM CREAF5 and CREAR5, 2.5 mM each deoxynucleotide triphosphate (dNTP), 100 ng of genomic DNA from A. citri, and the reaction buffer supplied with the PCR DIG Probe Synthesis Kit (Roche, Mannheim, Germany) were used. DIG-labeled PCR products were separated by agarose gel electrophoresis (1% agarose), and the PCR products were gel-purified using a NucleoSpin Extract II kit (Macherey-Nagel, Düren, Germany) according to the manufacturer’s instructions. For northern blot analysis, another PCR DIG probe was used for the entire coding region of CreA. PCR was performed in a Gene Amp PCR System 9700 with primers CREAF5 (forward primer) and CREAR5 (reverse primer), following the manufacturer’s instructions. For northern blot analysis, another PCR DIG probe for the entire coding region of CreA was also prepared with primers CreAF (5′-ATGCAATCCAACTCTGCTCT-3′) and CreAR (without TAA) (5′-GGTGCGATGTGACAGGTCC-3′) and an Acpg1 PCR probe with PG#6 (5′-CAATGGTGGCCTAAGGC-3′) and PG#8 (5′-A CGAACACTTTGGAACGC-3′) (17), following the manufacturer’s instructions.

Genomic DNA (5 µg) was digested with 10 units of restriction enzyme EcoRI, SacI, or HindIII according to the manufacturer’s instructions (Takara) and fractionated in 1% agarose gels. Total RNA was extracted using the RNasy Plant Mini Kit (Qiagen, Valencia, CA) from mycelial mats harvested after 10 or 25 days of incubation. Total RNA was loaded on 1% agarose-formaldehyde gels (5 or 10 µg/lane) and transferred to a Hybond N+ membrane (GE Healthcare Bio-Sciences, NJ) by capillary transfer. Total RNA on the membrane was stained with 0.2% (wt/vol) methylene blue in 0.3 M sodium acetate (pH 5.2) and destained in 20% (vol/vol) ethanol. The rRNAs were stained with methylene blue to confirm equal RNA loading. Hybridization was performed at 68°C overnight with the PCR DIG probe (100 ng/ml) for both Southern and northern blots, and was visualized with anti-DIG antibody-alkaline phosphatase and CSPD and exposing the membranes to X-ray films (Fujiﬁlm RX-U, Tokyo).

**Preparations of plasmid construct and AcCreA overexpression mutant.** The overexpression vector pAOEIK was produced by ligating the cassette of a trpC promoter (20) and trpC terminator (24) of Aspergillus nidulans, which were recovered from pSH75 (19), into PCR-script SK Amp (+) (Stratagene, La Jolla, CA) at BamHI and XhoI sites. A full-length AcCreA sequence was amplified with the primer set EcoRIaf+C reAF (5′-GGAATTCATGCAATCTAACTCTTGGCTCAGCAAGTCGACAGGT-3′) and CreAR+EcoR I (5′-CCGAATTCTTTAAGTGG-3′) (Table 1). Insertion of the amplified

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence</th>
<th>Purpose</th>
<th>Reference</th>
</tr>
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<td>CREAF5</td>
<td>GARAARCNCAYGCGNTG</td>
<td>Degenerate PCR, RACE, probe preparation</td>
<td>This study</td>
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<td>Clontech kit</td>
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<tr>
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<td>This study</td>
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<tr>
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<td>This study</td>
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<td>Insertion check</td>
<td>This study</td>
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<td>Probe preparation</td>
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<tr>
<td>CreAF (ATG)</td>
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<td>Isshiki et al. 2001 (17)</td>
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<td>PG#6</td>
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<td>Probe preparation</td>
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<td>PG#8</td>
<td>ACAGAACCCTTTGGAACGC</td>
<td>Probe preparation</td>
<td>Isshiki et al. 2001 (17)</td>
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a PCR = polymerase chain reaction and RACE = rapid amplification of cDNA ends.
AcCreA at the EcoRI site in the overexpression vector, resulting in the construction of pAOEIK-AcCreA, was confirmed by PCR analysis with primer set PtpCF2 (5'-GTGATACCTTCTTAAAGT-TCGCCC-3') (Table 1) and CreAR+EcOR3 and sequencing the product.

Protoplasts of Alternaria citri were prepared with Lysing enzyme (Sigma-Aldrich) at 10 mg/ml and Kitalase (Wako, Tokyo) product. PCR amplification with primer set CREAF5 and CREAR5 (Table 1) and genomic DNA of regions of these fungal creA sequences (Fig. 1). PCR amplification with degenerate PCR primers were designed from the conserved sequences of creA from phytopathogenic fungi were aligned by CLUSTAL W (36), and identical residues were indicated with asterisks. The two Cys2His2 zinc finger regions (3,31) are underlined with numbers 1 and 2. Two boxes with arrows indicate the annealing regions for degenerate primers CREAF5 and CREAR5 (reverse primer) (Table 1) and genomic DNA of Alternaria citri as a template gave a 703-bp product. The nucleotide sequence of the product showed a high degree of sequence similarity to known fungal creA genes. RACE was used to clone the full-length gene, were designed. PCR amplification with these primers and sequencing the product confirmed the full sequence of A. citri creA cDNA, designated AcCreA (databank accession no. AB267479). This cDNA consists of 1,346 bp containing a 1,290-bp open reading frame flanked by a 21-bp 5' UTR sequence and a 35-bp 3' UTR. The primer UCRAF1 and UCRA3 (Table 1) amplified a 1,395-bp product from genomic DNA, and comparison of the two sequences from cDNA and genomic DNA identified a 49-bp intron at nucleotide position 339 to 387 in the genomic sequence. Except for the intron region, the sequences of PCR products using the primers described above from both total RNA and genomic DNA were identical.

The creA gene of A. citri consisted of a 1,290-bp open reading frame encoding a predicted protein product of 430 amino acids (Fig. 1) with an estimated molecular mass of 46.4 kDa and a pI of 9.59. CLUSTAL W analysis showed 57, 52, 92, 52, and 50% amino acid sequence identity with the sequences of CreA from Aspergillus nidulans (12) (databank accession no. L03563), Cochliobolus carbonum (37) (accession no. L03563), Gibberella fujikuroi (37) (accession no. Y16625), and Sclerotinia sclerotiorum (40) (accession no. L03563), respectively. The deduced amino acid sequences of creA from A. citri, the gene, were designed. PCR amplification with these primers and sequencing the product confirmed the full sequence of A. citri creA cDNA, designated AcCreA (databank accession no. AB267479). This cDNA consists of 1,346 bp containing a 1,290-bp open reading frame flanked by a 21-bp 5' UTR sequence and a 35-bp 3' UTR. The primer UCRAF1 and UCRA3 (Table 1) amplified a 1,395-bp product from genomic DNA, and comparison of the two sequences from cDNA and genomic DNA identified a 49-bp intron at nucleotide position 339 to 387 in the genomic sequence. Except for the intron region, the sequences of PCR products using the primers described above from both total RNA and genomic DNA were identical.

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B. cinerea (39) (accession no. Y16625), C. carbonum (37) (accession no. AF306571), G. fujikuroi (39) (accession no. Y16626), and S. sclerotiorum (40) (accession no. AJ000976), respectively (Fig. 1). Sequence analysis using the PROSITE program (15) identified two Cys2His2-type zinc fingers at amino acid positions 68 to 88 and 96 to 118, indicating that this protein has a putative DNA binding function (Fig. 1). AcCreA also possessed a nuclear localization signal (14) of PNSRRGK, at amino acid position 121 to 127 (Fig. 1).

Hybridization of a probe corresponding to the internal part of the AcCreA gene using genomic DNA of Alternaria citri digested with EcoRI, SacI, or HindIII resulted in the appearance a single band for the EcoRI-digested genome and several additional bands in the other two enzyme-digested genomes (Fig. 2A). These restriction enzymes were used because the sequence of the cloned AcCreA gene does not have sites for EcoRI, SacI, or HindIII.

To confirm overexpression of the AcCreA gene, AcCreA transcripts were detected by northern blot analysis. Northern blots identified transcripts with the expected size of 1,300 bp in the total RNA from AcOEC2 grown in both PDB and pectin media, whereas only traces of the transcripts were detected in total RNA of the wild type grown in PDB or pectin medium (Fig. 2D).

**Characterization of CreA overexpression mutants.** The CreA overexpression mutant AcOEC2 was able to utilize pectin as a sole carbon source for growth in a liquid medium. A block of AcOEC2 or wild-type A. citri mycelia was placed on the pectin medium with a sterilized piece of the internal surface of citrus peel or the citrus juice sac area. The growth of this mutant on pectin medium was slightly slower than that of the wild type, and the growth on citrus peel and citrus juice sac area of AcOEC2 was similar to that of the wild-type strain (Fig. 3A). EndoPG activity measured by the decrease in relative viscosity of a 1.5% (wt/vol) solution (pH 5.0) of polygalacturonic acid also showed normal endoPG production in the culture filtrates from the AcCreA overexpression mutant AcOEC2 similar to that of the wild type (Fig. 3B). AcOEC2 sporulated as well as the corresponding wild types, and there were no differences in spore germination, appressorium formation, or infection hypha formation on cellulose membranes (data not shown). Of 10 transformants obtained, 3 were examined in the same manner and all showed similar results, and one of the three transformants, AcOEC2, was further examined.

**Fig. 2.** Construction of an overexpression mutant of AcCreA from Alternaria citri. A. Examination of copy number of the AcCreA gene in the A. citri genome. Southern blot analysis of total genomic DNA from A. citri digested with EcoRI (lane 1), SacI (lane 2), or HindIII (lane 3) using a AcCreA-specific probe for the internal region of this gene. B. Map of plasmid construct of AcCreA overexpression vector pAOEIK-AcCreA. Arrows indicate the annealing sites for polymerase chain reaction (PCR) primers of PtpCF2 and CreAR+EcoR3 (CE). C. PCR analysis of insertion of AcCreA overexpression vector pAOEIK-AcCreA in the A. citri genome. Genomic DNA from wild-type A. citri (WT) or AcCreA overexpression mutant AcOEC2 (OEC2) was used for PCR amplification with primer sets of PtpCF2 and CreAR+EcoR3 (panel B) for detection of pAOEIK-AcCreA insertion. D. Confirmation of overexpression of AcCreA in AcOEC2 mutant. Northern blot analysis of the AcCreA gene was performed with total RNAs isolated from mycelial mats of wild-type A. citri (WT) or of the AcCreA overexpression mutant AcOEC2 (OEC2), from 10-day-old cultures in potato dextrose broth (PDB), or from 25-day-old cultures in pectin medium. Equality of RNA loading in each lane was estimated by rRNA stained with methylene blue.

**Fig. 3.** Growth and endopolygalacturonomase (endoPG) production by overexpression mutant AcOEC2 in pectin medium and plant materials. A. Block of mycelia of wild-type Alternaria citri (WT) or AcCreA overexpression mutant AcOEC2 (OEC2) was placed on pectin medium and plant materials (Peel or Juice sac area). Typical growth after a 5-day incubation is shown. B. EndoPG production by A. citri (WT) or AcCreA overexpression mutant AcOEC2 (OEC2). Total enzyme activity produced by either fungus in 50 ml of pectin medium after a 25-day incubation at 24°C was measured by a relative viscosity assay. One unit was defined as the activity that reduces 50% of viscosity of a 1.5% polygalacturonic acid solution (pH 5.0) at 37°C for 5 min, as described previously (16). Data indicate the mean of five independent repeats with standard deviation values.
EndoPG gene (Acpg1) expression and endoPG production were examined under a condition that induced carbon catabolite repression: addition of 2% glucose (17,18,26). Both the wild type and AcOEC2 were cultured for 10 days in liquid medium containing 1% (wt/vol) pectin with or without 2% (wt/vol) glucose, and the transcript accumulation of AcCreA and Acpg1 in the total RNA isolated from the mycelial mats as well as endoPG production in the culture filtrates were analyzed (Fig. 4). Transcription of Acpg1 and endoPG production of wild-type *A. citri* were repressed when 2% (wt/vol) glucose was added to the pectin medium, as described previously (17,18,26) (Fig. 4). However, transcripts of *Acpg1* were detected and endoPG also was produced in the culture filtrates from AcOEC2, and the repression observed in the wild type by addition of 2% (wt/vol) glucose was overcome by the AcCreA overexpression mutant AcOEC2 (Fig. 4). Transcription of AcCreA itself was independent of the addition of glucose, and the expression was not induced in either the wild type or AcOEC2 by glucose addition (Fig. 4A).

**Symptom development in citrus due to AcCreA overexpression mutant.** Spore suspensions (1 × 10^5 spores per 100 µl) of wild-type *A. citri* or the AcCreA overexpression mutant AcOEC2 were injected directly into the central axis of sterilized citrus fruit. After 2 weeks, the wild-type strain had not induced rosetting except at the injection site, whereas AcOEC2 induced black rot symptoms in both the central axis and juice sac areas (Fig. 5). The wild-type strain also induced severe black rotting after 4 weeks, but the development of roting was limited to the central axis area and the black rot symptom had not expanded into the juice sac area (Fig. 5). Unlike the typical symptoms caused by the wild type, AcOEC2 expressing AcCreA showed continuous expansion of black rot symptoms in both the central axis and juice sac areas (Fig. 5). Similar results were found in both the Washington navel orange and Hassaku orange.

**DISCUSSION**

Fungal CreA is a cis-acting zinc finger repressor protein involved in carbon catabolite repression. CreA is currently the only well-characterized factor involved in fungal carbon catabolite repression, but its role in the pathogenesis of fungal phytopathogens has never been examined. Some studies have reported the involvement of the carbon catabolite repressor, CreA, in the regulation of fungal-secreted cell wall-degrading enzymes (2,11,22,27–29), and the overexpression of the creA gene resulted in a slightly higher repression of α-amylase production in *Aspergillus nidulans* (1). Furthermore, mutation of creA in *A. nidulans* resulted in considerably elevated levels of *xlbB* mRNA (28). However, none of these studies linked the functions of CreA with regulation of the pathogenicity of the producing fungus.

To examine the relationship between the CreA-mediated carbon catabolite repression and the pathogenesis of phytopathogenic fungi, we made an overexpression mutant of AcCreA instead of a disruption mutant of this gene in *Alternaria citri* because a genomic Southern analysis of AcCreA in *A. citri* indicated the presence of multiple copies of this gene and also because disruption of the gene encoding CreA in *Aspergillus nidulans* produced a partially lethal phenotype or showed some defects on its growth (12,35). Any defects on the growth of microbe are not suitable for use in studies of plant–microbe interactions because the defects will complicate the evaluation of the changes observed in plant–microbe interactions.

The product of this gene, AcCreA, is similar to other fungal CreAs, which have a signature zinc finger domain of the Cys2His2 type (3,31), and is expected to bind to the promoter region of target genes and downregulate gene expression, affecting secondary metabolites similar to the functions of CreA in other fungi (44). However, overexpression of the AcCreA gene in *Alternaria citri* unexpectedly led to a loss of the carbon catabolite repression function in the regulation of *Acpg1* gene expression. Direct evidence of AcCreA binding to the *Acpg1* promoter was not obtained because quantitative expression using prokaryotic systems damaged *Escherichia coli* cells and did not reproduce this regulatory protein (data not shown); thus, regular binding experiments for a regulatory protein, such as a gel shift assay, were impossible to perform without a large amount of AcCreA protein. However, the promoter sequence of the *Acpg1* gene has five typical binding sites (5′-SYGGRG-3′) for CreA (10,13) in the 813 bp upstream of the translation start site, and various deletion experiments of the promoter region found that the loss of the CreA binding site at -79 resulted in a loss of carbon catabolite repression on the transcription initiation function of the *Acpg1* promoter, clearly indicating the involvement of CreA in the regulation of *Acpg1* expression (26). Therefore, the effect of AcCreA on the endoPG of *A. citri* was monitored by both transcription of *Acpg1* and production of Acpg1 in culture filtrates. Unlike the repression by the wild type, transcription of *Acpg1* in the AcCreA overexpression mutant AcOEC2 was not repressed by the addition of glucose and, thus, the production of endoPG in the culture filtrates.
also was not inhibited by carbon catabolite repression. Moreover, transcription of AcCreA itself was not significantly induced by addition of glucose, similar to results obtained for G. fujikuroi, B. cinerea, and S. sclerotiorum, in which creA transcription was independent of the addition of various carbon sources (39,41). Under carbon catabolite repression, multiple genes encoding products that metabolize carbon sources other than glucose generally are known to be suppressed, but a relationship between the CreA-mediated catabolite repression and regulation of endoPG gene expression among fungal phytopathogens was reported only for A. citri and S. sclerotiorum (26,29,41). CreA was suggested to be involved in the regulation of the pg1 gene from S. sclerotiorum by binding to the promoter sequence of pg1 (29), but the role of CreA in the pathogenesis of S. sclerotiorum was unknown.

The eradication of carbon catabolite repression by AcCreA overexpression that we found was directly linked to dramatic changes in symptom induction of A. citri in citrus fruit. AcOEC2 induced more severe symptoms at an earlier stage of infection, and rotting was observed in the juice sac area of citrus fruit, a symptom which the wild-type A. citri does not cause. As described earlier, black rot induction of this pathogen depends upon the production of an extracellular endoPG during the infection stage. After disruption of the Acpg1 gene from A. citri (17), soft rot symptoms in the central axis area were reduced by 85% as a result of the inhibition of penetration and maceration of citrus tissue (17). Interestingly, the green fluorescence of A. citri transformant EPG7 carrying a GFP gene under control of the Acpg1 gene promoter of A. citri was induced by pectin in the peel during the infection stage, but repressed completely in the juice sac area, and the repression was expected to be caused by carbon catabolite repression by sugars in the juice (18). Our transformants containing constructs that fused CreA-binding site-deleted Acpg1 promoters with the GFP reporter gene showed interesting phenotypes. Transformants with construct PGPDL4 with a –401 to –813 deletion had both substrate induction and catabolite repression, whereas transformants with PGPDLS5 with an additional deletion from –1 to –84, including one putative CreA-binding site, had substrate induction and loss of catabolite repression (26). Green fluorescence of transformants with PGPDL4 was induced in the peel by pectin but repressed completely in the juice sac area of citrus fruit, but transformants with PGPDLS5 fluoresced green in both the peel and juice sac area, again indicating that the repression of A. citri endoPG gene of Acpg1 in the juice sac area is under the regulation of carbon catabolite repression (26). AcOEC2 caused symptoms and rotting in the juice sac area, indicating that the repression of Acpg1 expression in the wild type of A. citri in the juice sac area is the cause of catabolite repression and, thus, AcCreA-mediated catabolite repression controls the virulence or infection behaviors of this pathogen. As far as we know, this is the first example explaining the mechanism of the regulation of endoPG gene expression of phytopathogenic fungi in different tissues of host plants during different phases of infection by CreA and catabolite repression, as well as defining the relationship between pathogenesis and CreA or catabolite repression.

The mechanism causing the loss of carbon catabolite repression by overexpression of AcCreA is not clear at this point. However, an overdose of AcCreA to the promoter of Acpg1 might act as a repressor of Acpg1 transcription instead of a repressor if the functional system of fungal CreA is similar to that of the yeast system. In yeast, the Cys2His2 zinc-finger-containing protein Mig1 binds to the promoters of several genes (6,43) and causes carbon catabolite repression, similar to the function of filamentous fungal CreAs (21,43). However, Mig1 has been known to recruit two other co-repressor proteins, Ssn6 and Tup1, and the complex of these three proteins inhibits the expression of genes encoding enzymes that catalyze other carbon sources (38,43).

Sn6 and Tup1 subunits are known to work as adaptor or effecter subunits, and are required for the repression function (30,43). Interestingly, Mig1 alone worked as a transcriptional activator instead of a repressor when Ssn6 and Tup1 were absent (38,43). If filamentous fungal CreA also includes a complex like the yeast Mig1 system for repression, overexpression of AcCreA might cause an imbalance in the subunit ratio and, thus, AcCreA alone caused activation the expression of Acpg1 instead of repressing it. Identification of functional or sequence homologues of Ssn6 and Tup1 from filamentous fungi will verify our prediction, and their identification might explain the mechanism of the loss of carbon catabolite repression by overexpression of AcCreA.

ACKNOWLEDGMENTS

This study was supported in part by grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan, the Japanese Society for the Promotion of Science, PROBRAIN, and PRESTO, Japan Science and Technology Agency. We thank T. Tsuge, Nagoya University, for providing transformation vector pSH75.

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