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

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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. How-

ever, 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 rotting 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.

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). In previous work, we purified endopolygalacturonases (endoPG) (EC 3.2.1.15) that can degrade pectic polysaccharide components in cell walls (16), and cloned the genes from two different species of *Alternaria* (17). An endoPG-disrupted mutant of *A. citri* had significantly reduced ability to cause black rot symptoms in citrus (17). More recently, we monitored the colonization of *A. citri* in citrus fruit tissues using mutants expressing a gene encoding green fluorescent protein (GFP) as a vital marker (18). Hyphae of the GFP transformant derived from the wild-type (G1) but not the transformant derived from its endoPG-disrupted mutant (GM4) penetrated the peel vertically, although the hyphae of both G1 and GM4 spread equally in the juice sac area when the mutants were injected directly into the central axis of fruit (18). The pattern of endoPG gene expression in various parts of citrus fruit also was examined using the *A. citri* mutant EPG7 carrying a GFP gene under the control of the 813-bp putative endoPG gene (*AcpG1*)

promoter region (18). The green fluorescence of EPG7 was induced by pectin in the peel but repressed completely in the juice sac area (18). The cause of repression in the juice sac area was expected to be carbon catabolite repression by sugars in the juice. In many organisms, glucose can repress multiple genes encoding products that metabolize carbon sources other than glucose, and several studies of yeast and filamentous fungi have revealed a putative mechanism for the glucose repression of eukaryotes that can be distinguished from the mechanism found in bacteria (30). A protein found to play roles in this carbon catabolite repression in *Aspergillus nidulans* was named a catabolite repression element (*creA*); it is a homolog of the yeast Mig1 and shares functions similar to those of Mig1 (1,12,43). *CreA* possesses a zinc finger domain of the Cys₂His₂ type which prevents transcription of several genes in the presence of glucose by sequence-specific binding to the promoters of the target genes (10,13,37). 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

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The nucleotide sequence data reported in this paper appears in the DNA Data Bank of Japan, EMBL, and GenBank sequence database under accession no. AB267479 for *AcCreA*.

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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 broth (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, CREAM5 (5'-GARAARCCNCAYGCNTG-3', forward primer) and CREAM5 (5'-GTRTGRTCNNGGNTNGG-3', reverse primer) (Table 1), designed from the conserved region of deduced amino acid sequences of CreAs 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) with *Taq* DNA polymerase (2.5 units; Takara, Shiga, Japan) at 95°C for 2 min for one cycle, and 30 cycles of 2 min at 95°C , 2 min at 50°C , and 1 min at 72°C , with 2 μM CREAM5 and CREAM5, 2.5 mM each deoxynucleotide triphosphate (dNTP), 100 ng of genomic DNA from *A. citri*, and the reaction buffer supplied with the *Taq* DNA polymerase (Takara).

Rapid amplification of cDNA ends (RACE) was used to obtain the full-length cDNAs using a Marathon cDNA Amplification Kit (Clontech, Palo Alto, CA). Total RNA (6 μg) was reverse transcribed with an oligo (dT) primer, and second-strand cDNA synthesis and ligation of adaptors was performed with the Marathon kit. Subsequent PCR was conducted with the Clontech Adapter Primer 1 (AP1) (5'-CCATCCTAATACGACTCACTATAGGGC-3') (Table 1) and either forward primer CREAM5 (3' RACE) or reverse primer CREAM5 (5' RACE). The products were subcloned into a pTBlue-2 T-vector (Novagen, Madison, WI). The sequences of 5' and 3' RACE products were obtained from both strands by the dideoxy chain termination method (33) with the use of an ABI PRISM Dye Termination Cycle Sequencing Ready Reaction Kit (Applied Biosystems) and an automated fluorescent DNA sequencer (Model 310; Applied Biosystems). Another set of PCR primers, UCREAF1 (forward primer, 5'-ACAGCGGGGACCCTCGGTCAC-3') (Table 1) and UCREAR3 (reverse primer, 5'-AG-

TTTGTCCATATCTAATCTCATCGCTTGC-3') (Table 1), designed from the upper end of the 5' RACE product and the untranslated region (UTR) downstream from the 3' RACE product was used to amplify the entire region of the *creA* gene from either genomic DNA or cDNA, under the PCR conditions as described for the amplification of the internal region above, except that the concentration of primers UCREAF1 and UCREAR3 was 1 μM each. DNA sequences were aligned with the CLUSTAL W program (36), and sequence analysis was performed using the PROSITE program (15), PSOERT program (25), SMART program (34), and GENETYX-MAC (Software Development Ltd., Tokyo). Homology analysis was performed using BLAST at the DNA Data Bank of Japan.

Blotting conditions. For Southern blot analysis, a PCR digoxigenin (DIG) probe was prepared from the *AcCreA* sequence in a pTBlue-2 T-vector as a template for labeling using the PCR DIG Probe Synthesis Kit (Roche, Mannheim, Germany) with primers CREAM5 (forward primer) and CREAM5 (reverse primer), following the manufacturer's instructions. For northern blot analysis, another PCR DIG probe for the entire coding region of *AcCreA* also was prepared with primers CreAF (ATG) (5'-ATGCAATCCAACTCTGCCTC-3') and CreAR (withoutTAA) (5'-GTTGCGATGTGACAGGTCCT-3') and an *AcpG1* PCR probe with PG#6 (5'-ACAATGGTTGCCTTAACT-3') and PG#8 (5'-ACGAACACTTGGTAACGC-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 RNeasy 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 $\mu\text{g}/\text{lane}$) and transferred to a Hybond N⁺ membrane (GE Healthcare Bio-Sciences, NJ) by capillary transfer. Total RNA on the membrane was stained with 0.02% (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 ligation of 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 *Bam*HI and *Xho*I sites. A full-length *AcCreA* sequence was amplified with the primer set EcoRI site+CreAF (5'-GGAATTCCATGCAATCCAACTCTGCCTCGACAGGT-3') and CreAR+EcoRI3 (5'-CCGGAATTCTTAGTTGCGATGTGACAGGTC-3') (Table 1). Insertion of the amplified

TABLE 1. Primers used in this study

Primer name	Sequence (5' to 3')	Purpose ^a	Reference
CREAF5	GARAARCCNCAYGCNTG	Degenerate PCR, RACE, probe preparation	This study
CREAR5	GTRTGRTCNNGGNTNGG	Degenerate PCR, RACE, probe preparation	This study
AP1	CCATCCTAATACGACTCACTATAGGGC	RACE	Clontech kit
UCREAF1	ACAGCGGGGACCCTCGGTCAC	Amplification of full-length <i>AcCreA</i>	This study
UCREAR3	AGTTTGTCCATATCTAATCTCATCGCTTGC	Amplification of full-length <i>AcCreA</i>	This study
EcoRI site+CreAF	GGAATTCCATGCAATCCAACTCTGCCTCGACAGGT	Subcloning to pAOEIK	This study
CreAR+EcoRI3	CCGGAATTCTTAGTTGCGATGTGACAGGTC	Subcloning to pAOEIK/insertion check	This study
PtpCF2	GTCATACCTTCTTAAGTTCCGC	Insertion check	This study
CreAF (ATG)	ATGCAATCCAACTCTGCCTC	Probe preparation	This study
CreAR (without TAA)	GTTGCGATGTGACAGGTCCT	Probe preparation	This study
PG#6	ACAATGGTTGCCTTAACT	Probe preparation	Isshiki et al. 2001 (17)
PG#8	ACGAACACTTGGTAACGC	Probe preparation	Isshiki et al. 2001 (17)

^a PCR = polymerase chain reaction and RACE = rapid amplification of cDNA ends.

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 *Eco*RI, *Sac*I, or *Hind*III resulted in the appearance a single band for the *Eco*RI-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 *Eco*RI, *Sac*I, or *Hind*III.

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 *Eco*RI (lane 1), *Sac*I (lane 2), or *Hind*III (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 PtrpCF2 and CreAR+EcoRI3 (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 PtrpCF2 and CreAR+EcoRI3 (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.

Construction of *CreA* overexpression mutants. Hygromycin-resistant transformants of *A. citri* were generated by co-transformation with the vector pSH75 and *AcCreA* overexpression vector pAOEIK-*AcCreA* (Fig. 2B). The overexpression vector pAOEIK was constructed in a PCR-Script SK (+) vector (Stratagene) with additions of the constitutive expression *trpC* promoter (20) and *trpC* terminator (24) of *Aspergillus nidulans*. Integration of pAOEIK-*AcCreA* into the *Alternaria citri* genome was confirmed by detection of a band of the expected size of 1,430 bp by PCR analysis (Fig. 2C).

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. 3. Growth and endopolygalacturonase (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 μ l) 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 rotting 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 rotting 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 *xlnB* 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

Fig. 4. Loss of carbon catabolite repression of endopolygalacturonase (endoPG) production in *AcCreA* overexpression mutant AcOEC2 of *Alternaria citri*. **A**, Northern blot analysis of *AcCreA* and endoPG genes in mycelial mats of 10-day-old cultures in pectin medium with or without addition of 2% (wt/vol) glucose. Equality of RNA loading in each lane was estimated by rRNA stained with methylene blue. **B**, Production of endoPG by AcOEC2 in the presence of glucose. PG activity was determined by a relative viscosity assay in 10-day-old culture filtrates of pectin medium with or without addition of 2% (wt/vol) glucose from the *A. citri* wild type (WT) or *AcCreA* overexpression mutant AcOEC2 (O2). Activity of the wild type grown in pectin medium without glucose was counted as 100%, and the relative percentage of each activity was calculated against it. Data indicate the mean of five independent repeats with standard deviation values.

Fig. 5. Evaluation of virulence of *AcCreA* overexpression mutant AcOEC2 of *Alternaria citri*. A spore suspension (1×10^5 spores/100 μ l) of the wild type (WT) or *AcCreA* overexpression mutant (AcOEC2) was injected directly into the central axis of sterilized navel or Hassaku oranges. The inoculated fruit were incubated for 2 weeks (navel orange) or 4 weeks (Hassaku orange).

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 *pgl* gene from *S. sclerotiorum* by binding to the promoter sequence of *pgl* (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 *Acpgl* 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 *Acpgl* 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 *Acpgl* promoters with the GFP reporter gene showed interesting phenotypes. Transformants with construct PGPD4 with a -401 to -813 deletion had both substrate induction and catabolite repression, whereas transformants with PGPD5 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 PGPD4 was induced in the peel by pectin but repressed completely in the juice sac area of citrus fruit, but transformants with PGPD5 fluoresced green in both the peel and juice sac area, again indicating that the repression of *A. citri* endoPG gene of *Acpgl* 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 *Acpgl* 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 *Acpgl* might act as an activator of *Acpgl* 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 catabolize other carbon sources (38,43).

Ssn6 and Tup1 subunits are known to work as adaptor or effector 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 *Acpgl* 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.

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