Cloning of a Gene Expressed during Appressorium Formation by *Colletotrichum gloeosporioides* and a Marked Decrease in Virulence by Disruption of This Gene

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Appressorium formation in germinating *Colletotrichum gloeosporioides* is induced by the surface wax of its host. One of the genes expressed uniquely in *C. gloeosporioides* during appressorium formation induced by the host signal has been designated cap20, and this gene and its cDNA were cloned and sequenced. Nucleotide sequences of both revealed an open reading frame that could encode a 183-­amino acid polypeptide that did not have significant homology with any known proteins. Reverse transcriptase–polymerase chain reaction detected cap20 gene transcripts at the infection front on the surface and within tomato fruits infected by *C. gloeosporioides*. Gene-disrupted mutants incapable of expressing cap20 showed a drastically decreased virulence on avocado and tomato fruits. These results suggest that cap20 plays a significant role in the infection of the host.

INTRODUCTION

Conidia from many phytopathogenic fungi germinate on the host surface, and the germ tube differentiates into an infection structure called the appressorium, which is essential for penetration into hosts (Emmet and Parberry, 1975; Heath and Heath, 1978; Staples and Macko, 1980; Staples and Hoch, 1987). Signals from the host plant are known to induce germination of the fungal spore and appressorium formation (Hoch and Staples, 1991). Certain physical features of the host surface are thought to trigger appressorium formation in some organisms (Dickinson, 1977, 1979; Staples et al., 1985; Hoch et al., 1987a), and some of the molecular events triggered by these physical signals have been studied (Bhairi et al., 1989; Xuei et al., 1992a, 1992b; Lee and Dean, 1993). In spite of the many indications that chemical signals from the host can induce appressorium formation, few cases of specific chemical signals involved in this process have been documented (Hoch and Staples, 1984; Edwards and Bowling, 1986; Hoch et al., 1987b). Recently, appressorium formation in *Colletotrichum gloeosporioides* was found to be induced specifically by the surface wax of its host (Podila et al., 1993). The molecular events triggered in the fungus by the chemical signals from the host and the genes uniquely involved in appressorium formation are not known. In this study, we report the discovery of a transcript uniquely expressed during appressorium formation in *C. gloeosporioides* induced by the host signal. We cloned and sequenced the cDNA and gene for this transcript. That this gene is involved in pathogenesis is suggested by the finding that the transcripts are found at the infection front in the host and by the observation that disruption of this gene causes a marked reduction in virulence on avocado and tomato fruits.

RESULTS

Isolation of cap20 cDNA and RNA Gel Blot Analysis of the cap20 Gene Expression

A subtracted cDNA library was constructed to enrich the cDNA associated with appressorium formation. The subtracted cDNA library was made by using the cDNA for nongerminating conidia to subtract the homologous population of DNA from the cDNAs for appressorium-forming conidia. The library was differentially screened with cDNA representing nongerminating, germinating, and appressorium-forming conidia, respectively. Screening of 4 × 10⁶ plaques yielded 82 individual clones that hybridized to the cDNA probes for only the appressorium-forming conidia. DNA gel blot analyses of these clones yielded four unique clones (Hwang and Kolattukudy, 1995). One of these clones, designated as cap20, contained one 1.1-kb insert.

RNA gel blot analysis showed that the cap20 transcript was highly expressed during appressorium formation. Total RNA
C. gloeosporioides

Gene and Its Transcript

To germinate and grow in yeast extract, and subsequently, the appressorium-forming conditions. Conidia were first allowed were not induced by avocado wax under nutrient-depleted non-

of this gene at significant levels (Figure 1A). cap20 transcripts were produced in the presence of host wax in yeast

extract, which does not allow appressorium formation. Total

scripts were produced in the presence of host wax in yeast

microorganisms.

Figure 1. Gel Blot Analyses of the cap20 cDNA and Gene.

(A) RNA gel blot showing induction of cap20 transcripts in

appressorium-forming conidia of C. gloeosporioides. The amounts (in micrograms) of total RNA isolated from conidia are shown. Conidia were treated as indicated for the times given. The blots were hybridized with 32P-labeled cap20 cDNA.

(B) DNA gel blot analysis of cap20 genomic DNA isolated from C. gloeosporioides. Genomic DNA (10 µg per lane) was digested with the indicated restriction enzymes, and the blots were hybridized with 32P-labeled cap20 cDNA. Length markers are indicated at left in kilobases.

isolated from nongerminating, germinating, and appressorium-

forming conidia was hybridized with the 32P-labeled insert fragment of the cap20 cDNA clone. A strong hybridization band at 1.3 kb was found with the RNA isolated from only the appressorium-forming conidia. We also tested whether the transcripts were produced in the presence of host wax in yeast extract, which does not allow appressorium formation. Total RNA from conidia incubated for 7 hr in yeast extract containing avocado wax suspension showed no hybridization with the cap20 cDNA probe, indicating that exposure to wax under non-appressorium-forming conditions did not cause expression of this gene at significant levels (Figure 1A). cap20 transcripts were not induced by avocado wax under nutrient-depleted non-appressorium-forming conditions. Conidia were first allowed to germinate and grow in yeast extract, and subsequently, the mycelia were treated with avocado wax in the presence or absence of yeast extract. Total RNA isolated from either showed no hybridization with the 32P-labeled insert fragment of cap20 cDNA.

DNA Gel Blot Analysis and Sequence of the cap20 Gene and Its Transcript

The genomic DNA isolated from C. gloeosporioides was digested with BamHI, EcoRI, HindIII, and SacI. Gel blots of the genomic DNA fragments from different digests were hybridized with the full-length insert fragment of the cap20 cDNA clone (Figure 1B). The results showed only one band in BamHI and EcoRI digests, but two bands were found in HindIII and SacI digests (as indicated in the following data, the open reading frame contains one SacI site and the intron contains one HindIII site). These results suggest that the genome of C. gloeosporioides probably contains one copy of the cap20 gene.

A genomic library of C. gloeosporioides, constructed in the λgt11 vector, was screened with the insert of cap20 cDNA. One genomic clone contained a 4-kb EcoRI fragment that hybridized with cap20 cDNA. This fragment was isolated, subcloned, and sequenced; the cDNA encoding CAP20 was also sequenced. The cDNA clone was composed of 1071 bp, which is close to the length of the transcript indicated by RNA gel blot analysis. We found one open reading frame starting with the first ATG codon at position 61 that would encode a 183-amino acid polypeptide with a molecular mass of 20,055 D (Figure 2). The amino acid sequence of this protein did not show significant homology with the sequences of other known proteins in the GenBank data base. Other possible reading frames initiating at other ATG codons would encode only small polypeptides with less than 80 amino acid residues. We tentatively concluded that the transcript is probably translated to yield a 20-kD protein. The 4-kb EcoRI fragment contained the entire cDNA with a 549-bp open reading frame identical to that found in the cDNA, with one interruption by a 59-bp intron. It also contained a 1.4-kb 5' upstream region containing a TATA box at position -445 and a CAAT box at position -468, as well as a 1.5-kb 3' downstream segment.

Expression of the CAP20 Protein in Escherichia coli

To determine whether the cap20 open reading frame is translated into a protein during appressorium formation and to locate the protein in the appressorium-forming conidia, an immunological approach with recombinant CAP20 protein was used. For this purpose, a polymerase chain reaction (PCR)-generated DNA segment containing the cap20 open reading frame was placed under the control of the T7 promoter in pET-19b and expressed in E. coli. SDS-PAGE analysis showed that the major protein in the induced cell extract was the 20-kD CAP20 protein. The recombinant CAP20 protein was purified with an Ni2+ affinity column, and polyclonal rabbit antibodies against it were prepared. The immunoblot showed strong cross-reactivity with the 20-kD protein (data not shown).

Protein Gel Blot Analysis of the CAP20 Protein in C. gloeosporioides during Appressorium Formation

The crude extracts from conidia, germinating conidia, and appressorium-forming conidia were analyzed by protein gel blot analysis with anti-CAP20 antiserum. Extracts of conidia
The putative TATA and CAAT box are shown by single and double underlines, respectively. The small letters represent the intron region. The ends of the cloned cDNA are indicated by arrows.

Figure 3. Protein Gel Blot Analyses of Total Proteins from C. gloeosporioides Conidia for the CAP20 Protein.

(A) Protein gel blot analyses of total proteins from germinating, non-germinating, and appressorium-forming conidia. The crude extract isolated from the conidia at the three indicated stages was subjected to SDS-PAGE. Anti-CAP20 antiserum and 125I-protein A were used to detect the proteins. App, appressorium.

(B) Time course of appearance of the CAP20 protein. Conidia exposed to avocado surface wax for the indicated periods were subjected to protein gel blot analysis as given in (A).

Molecular mass markers are given at left in kilodaltons.
Figure 4. RT-PCR Detection of cap20 Transcripts in Tomato Fruit Tissue Infected with Wild-Type C. gloeosporioides.

(A) DNA gel blot of the PCR product. Total RNA from the outermost layer of tomato fruit (I) and successive deeper layers of the fruit under the inoculated area (II) and (III) was used for RT-PCR, and the products were subjected to DNA gel blot with $^{32}$P-labeled cap20 cDNA as the probe.

(B) Ethidium bromide-stained RT-PCR products. RNA from the fruit layers indicated in (A) was used for RT-PCR, and the ethidium bromide-stained gel was photographed under UV light. (I), (II), and (III) are the same as given in (A). The expected length of the cap20 RT-PCR product is 730 bp.

In both (A) and (B), 1, 2, and 6 denote the number of days after inoculation.

In Vivo Expression of cap20 in Infected Tomato Fruit

To determine whether cap20 is expressed by C. gloeosporioides during the infection of its host, we placed conidia of C. gloeosporioides on ripening tomato surface, and infection was allowed to proceed in a humid atmosphere. A sensitive reverse transcriptase (RT)-PCR method (Chelly et al., 1988) was used to test for the presence of cap20 transcripts in the various layers of the fruit as infection proceeded into the fruit. As shown in Figure 4B, the cap20 transcript was detected 1 and 2 days after inoculation in the outer layer containing the cuticle/epidermis of the tomato fruit but not in the underlying tissue. However, when the disease lesions were visible after 6 days, the cap20 transcript was not detectable in the tomato cuticle/epidermis layer or the immediately underlying tissue. On the other hand, after the infection front, reaching deeper into the fruit, the cap20 transcript was detected by RT-PCR. The identity of the PCR products was confirmed by DNA gel blot hybridization (Figure 4A) and by DNA sequencing, which showed that the sequence of the PCR product was identical to that of the cDNA for the cap20 transcript found in appressorium-forming fungal conidia. Thus, the cap20 gene is expressed in vivo during infection of the host by C. gloeosporioides.

Generation of the cap20-Disrupted Mutants

To construct a vector for cap20 disruption, a 4-kb genomic fragment containing the cap20 gene was subcloned into pUC18 with a deleted BamHI site. The 2.4-kb hygromycin gene fused to a C. heterostrophus promoter was ligated into the 4-kb fragment at the Hpal site so that the open reading frame of cap20 was interrupted; the final construct was designated as pD20 (Figure 5). Hygromycin-resistant transformants generated with this vector were examined by DNA gel blot hybridization. When HindIII-digested genomic DNA preparations from 42 such transformants were subjected to DNA gel blot analysis with cap20 cDNA as the probe, all but four gave hybridization bands.

Figure 5. Schematic Presentation of the Strategy Used for cap20 Gene Disruption in C. gloeosporioides.

The C. heterostrophus promoter (Coc. prom.) fused to the hygromycin resistance (Hyg.) gene interrupted the open reading frame of the cap20 gene. Open boxes represent the 5' and 3' flanking regions of the cap20 gene. Black boxes represent the open reading frame of the cap20 gene. The thin lines represent the intron region. H3, HindIII site; X, genetic cross-over.
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![Image of gel blot analysis](image)

Figure 6. Gel Blot Analyses of cap20-Disrupted Mutants of C. gloeosporioides.

(A) DNA blot analysis of genomic DNA isolated from the wild type (wt) and cap20-disrupted mutants (indicated above the gels) of C. gloeosporioides. Genomic DNA (10 μg per lane) was digested with HindIII and hybridized with 32P-labeled cap20 cDNA (left) or the hygromycin resistance gene (right). The length markers are indicated at left in kilobases.

(B) Protein blot analysis of total proteins from the wild type and cap20-disrupted mutants of C. gloeosporioides to detect the presence of the CAP20 protein. The crude extracts of conidia and appressorium-forming conidia from the wild type (wt) and mutants 4 and 5 indicated in (A) were subjected to protein gel blot analysis with anti-CAP20 antiserum and 125I-protein A for protein detection. The size markers are indicated at left in kilodaltons.

Tests for Pathogenicity of the cap20-Disrupted Mutants on Avocado and Tomato Fruits

Although cap20 gene disruption did not seem to affect appressorium formation adversely, these appressoria might not be fully functional in infection. To test for this possibility, the pathogenicity of the conidia of the cap20-disrupted mutants was compared with that of the conidia of the wild type. When the same number of conidia were placed on avocado fruits, the wild-type conidia caused lesion development, whereas with the conidia of D4 and D5, no lesion was found, although in some cases surface growth of aerial mycelia was observed. When the wild type had shown clear symptoms of infection, the fruits were cut longitudinally through the region where conidia were placed and the cross-sections were examined for lesions. The fruits inoculated with wild-type conidia showed clear infection lesions that had progressed deep into the fruit. No lesions were observed with the conidia of the cap20-disrupted mutants (Figure 7, top). On the other hand, both the wild type and the cap20-disrupted mutant D5 showed similar lesion development when spore suspensions were placed on the surface of avocado fruits with a pin prick (data not shown).

The conidia of C. gloeosporioides have been shown to form appressoria and cause infection symptoms on ripening tomato fruits (Flaishman and Kolattukudy, 1994). Hence, we tested whether disruption of the cap20 gene affected the pathogenicity on ripening tomato fruits. Tomato fruits inoculated with the wild type showed infection symptoms, whereas fruits inoculated with the two cap20-disrupted mutants showed no infection symptom, although aerial mycelia were found on the surface of some fruits. When the outermost cuticle-containing peel (~1 mm) was removed, fungal infection was visible in the fruits inoculated with wild-type conidia, whereas no sign of infection was found in the fruits inoculated with the D4 or D5 conidia (Figure 7, bottom). Removal of additional layers underlying the infected area showed that infection had proceeded quite deep into the fruits.
Tests for Pathogenicity of the Wild Type and cap20-Disrupted Mutants of C. gloeosporioides on Avocado and Tomato Fruits.

Conidia ($10^4$) of the wild type or the mutants were placed in a 1-cm$^2$ area of the fruit surface and incubated under high humidity until the wild type resulted in obvious lesion formation. The cross-sections of avocado fruits (top) through the inoculation area (arrows) are shown. The top layer of the tomato fruits (bottom) was removed to expose the internal tissue. D4 and D5 mutants are cap20-disrupted mutants 4 and 5 indicated in Figure 6.

DISCUSSION

The fungal genes involved in appressorium formation triggered by chemical signals from the host plant have not been identified. In this article, we describe one such gene induced by the surface wax of the host organ. The subtractive approach we used yielded many clones, but only a few represented the transcripts uniquely expressed during the appressorium formation induced by the host signal. cap20 was found to be uniquely expressed during appressorium formation, as indicated by the observation that this transcript was found only during appressorium formation and not during germination. RNA gel blot analysis clearly showed that the transcript was not induced by wax under non-appressorium-forming conditions. The host wax did not induce the expression of cap20 under nutrient-rich conditions that did not allow appressorium formation. The nutrient-rich condition could have caused a catabolite repression of the cap20 gene. However, even when the germinated conidia were washed free of nutrients and placed in nutrient-free medium with avocado wax, the cap20 gene was not expressed. Thus, cap20 clearly appears to be a gene whose expression is induced by the host wax uniquely during appressorium formation.

That the cloned cap20 cDNA truly represented a transcript was demonstrated by the finding that the nucleotide sequence of this cDNA matched the sequence of a DNA segment cloned from the genomic DNA except for the presence of a 59-bp intron. This nucleotide sequence or the deduced amino acid sequence did not show significant homology with any known gene or protein. The mpg7 gene from Magnaporthe grisea that appears to be involved in appressorium formation (Talbot et al., 1993) did not show homology with cap20. Obviously, appressorium formation would require the expression of a set of genes: the present cap20 gene, three genes cloned from C. gloeosporioides, cap3, cap5, and cap22 (Hwang and...
Kolattukudy, 1995), and the mpgl gene from M. grisea are the members of this group cloned and identified so far.

To determine whether the open reading frame found in the cap20 gene is actually expressed in the fungus during appressorium formation, we used an indirect immunological approach. The open reading frame found in cap20 cDNA could be readily expressed in E. coli, yielding a protein of the expected size (20 kD). Antibodies prepared against this recombinant protein showed cross-reactivity with proteins of appressorium-forming conidia. Protein gel blots showed that the appressorium-forming conidia had the highest level of this protein, although the nongerminating conidia also showed a low level of the cross-reacting protein. Whether this low level represents a background level found in the conidia or the presence of a low proportion of appressorium-forming conidia in the spore preparation used in such experiments is not clear. In any case, the CAP20 protein was not induced in C. gloeosporioides by the host wax under non-appressorium-forming conditions. These results strongly suggest that cap20 expression is most probably associated with appressorium formation.

Because no obvious functional role could be deduced from the structure of the protein, we thought disruption of the gene might reveal some biological consequence that could give clues as to the function of cap20. Therefore, C. gloeosporioides was transformed with a vector containing the cap20 gene disrupted by the presence of the hygromycin resistance gene. Of the 42 stable transformants examined, four revealed disruption of cap20. DNA gel blot analysis revealed cap20 disruption, and protein gel blot analysis confirmed that these transformants did not produce immunologically cross-reacting CAP20 protein. The conidia of these transformants germinated normally and differentiated into appressoria when treated with wax. Simple light microscopic examination showed that the appressoria formed by these conidia were normal in gross appearance; no morphological abnormality could be detected in these appressoria. There was also no discernible quantitative difference in the wax inducibility of appressorium formation. Spores of the gene-disrupted mutants germinated and formed normal-looking appressoria not only on glass surfaces coated with host wax, but also on the host. Light microscopic examination of the spores placed on tomato fruit surface could not detect any difference in germination and appressorium formation between the wild type and the two cap20-disrupted mutants; all of them germinated and formed normal-looking appressoria on the fruits. Thus, structural changes that might have resulted from the lack of the CAP20 protein were not manifested in gross morphological alterations. In M. grisea, disruption of a gene thought to be involved in appressorium formation also did not produce morphological abnormalities but showed a quantitative difference (Talbot et al., 1993). In our study, no such difference in appressorium formation was detected.

Even though the lack of CAP20 did not result in an obvious abnormality in appressorium formation, a functional abnormality might be revealed in the pathogenicity. To test for this possibility, conidia from cap20-disrupted mutants and wild-type conidia were placed on the surface of intact avocado fruits and lesion development was monitored. Whereas the wild type showed normal lesion development, cap20-disrupted mutants failed to infect fruits, clearly demonstrating a functional role for cap20 in pathogenesis. Because the presence of C. gloeosporioides has also been shown to result in lesions on ripening tomato fruits, we tested the pathogenicity of the wild type and cap20-disrupted mutants on this alternative host. Whereas the wild type penetrated deeply into the fruits and resulted in lesions, cap20-disrupted mutants were unable to penetrate into the fruit but sometimes grew on the fruit surface only. Examination of the tissue underlying the cuticle clearly showed the deep penetration of the wild type, with no indication of penetration by the mutants. That the function of the cap20 product is in the penetration process was further suggested by our observation that on avocado fruits with breached cuticle/cell wall barrier, both the wild type and the cap20-disrupted D5 mutant formed similar infection lesions.

If and where cap20 is expressed during infection of a host might give clues about the functional involvement of this gene product in infection. RT-PCR showed that cap20 was expressed during the infection of tomato fruits by C. gloeosporioides. During the early part of infection, the transcripts were confined to the outer segment of the fruit, where the germinating conidia were differentiating to form appressoria that were allowing penetration through the outer barriers of the fruit. As penetration and infection proceeded into the fruits, the outer layer no longer constituted the infection front containing penetrating structures, and therefore cap20 transcripts could not be found. RT-PCR could not detect cap20 transcripts in the layers through which the fungus had already passed. However, cap20 transcripts were detected in the deeper layer representing the infection front. These results suggest that cap20 function might be at the advancing front of the fungal invasion in addition to its function in the original penetration involving well-known appressorium formation. It is possible that the fungal mycelia behind the infection front were killed by toxic compounds released by plant cell lysis and therefore did not contain cap20 transcripts. It is also possible that cap20 expression at the infection front involves appressorium formation within the tissue. Our cytochemical examination detected structures that could be internal appressoria at the infection front (data not shown). Appressorium formation in the interior of plants has been previously reported (Freeman and Rodriguez, 1993). If what we observed are internal appressoria, cap20 expression might be associated with such a penetration structure. The marked decrease in virulence caused by cap20 disruption supports the conclusion that the CAP20 protein is necessary to make a functional penetration structure. However, the mechanism by which cap20 helps to make the structure functional remains to be elucidated. Identification of the genes essential for the fungal differentiation process necessary for infection is only beginning. A hydrophobin-type protein (Templeton et al., 1994) was reported to be necessary for infection by M. grisea (Talbot et al., 1993). Our results show that other proteins uniquely expressed during appressorium formation may be essential for fungal infection. It is possible that such genes and/or their
products will offer novel targets suitable for intervention to protect plants from fungal infection.

METHODS

Isolation of Avocado Wax Extract and Avocado Fruit Homogenate

The surface wax of avocado fruit was isolated as described previously (Podila et al., 1993), and wax suspension was prepared by sonication of the wax in sterile water (1 mg/mL) for 3 to 5 min with a Sonifier Model 250 (Branson Ultrasonic, Danbury, CT). The final concentration of wax suspension was adjusted to 0.005% (w/v). The mesocarp of avocado fruit was homogenized for 1 to 2 min, and the homogenate was stored at -80°C.

Organism and Culture Conditions

Colletotrichum gloeosporioides, isolated from avocado, was provided by D. Prusky (Volcani Center, Bet Dagan, Israel). Cultures were maintained at 25°C on potato dextrose agar supplemented with 1% (w/v) water. The conidial suspension was filtered through two layers of avocado fruit homogenate. Conidia were obtained by gently scraping the dishes with a rubber policeman (Fischer Scientific, Cincinnati, OH) and recovered by centrifugation at 12,000 × g for 15 min. The number of conidia in the suspension was adjusted to 6 × 10^6 conidia per mL.

Induction of Germination and Appressorium Formation

Conidia were harvested from 5- to 7-day-old cultures. For RNA isolation, each Petri dish (10 × 150 mm) containing 40 to 45 mL of 0.005% (w/v) wax suspension and 5 × 10^6 conidia was incubated at 25°C for 3 to 4 hr, and the conidia (appressorium forming) were harvested. To obtain germination without appressorium formation (germinating), the same conditions as that used for appressorium formation were used except that 1% yeast extract was used instead of wax extract. Similar incubation in sterile distilled water resulted in no germination (nongerminating).

Isolation of Total RNA

Conidia from different treatment regimes were harvested by scraping them off the Petri dishes with a rubber policeman (Fischer Scientific, Cincinnati, OH) and recovered by centrifugation at 12,000 × g for 15 min. The conidia were suspended in guanidine isothiocyanate solution (5 M guanidine isothiocyanate, 50 mM Tris-Cl, pH 7.5, 10 mM Na2EDTA, pH 8.0, 5% β-mercaptoethanol) and disrupted for 60 to 90 sec with glass beads in a vortex mixer, and total RNA was isolated (Ausubel et al., 1992a). Because the appressorium-forming conidia strongly adhered to the dishes, a rubber policeman had to be used, and the damage to the tissue associated with this process resulted in a low recovery of RNA. In a typical experiment, 200 Petri dishes (10 × 150 mm), each containing 5 × 10^6 conidia, were incubated with wax suspension.

Construction of a Subtracted cDNA Library

The poly(A)^+ mRNA was isolated from the total RNA as described previously (Maniatis et al., 1982). Double-stranded cDNA was synthesized from the poly(A)^+ mRNA of nongerminating conidia and appressorium-forming conidia by using a cDNA synthesis kit (Invitrogen, San Diego, CA). After the second-strand cDNA synthesis, the double-stranded cDNA was phenol–chloroform extracted, ethanol precipitated, and resuspended in water. The cDNA synthesized from the nongerminating poly(A)^+ mRNA was digested with Real and Alul to give small blunt-ended fragments. The cDNA synthesized from the appressorium-forming poly(A)^+ mRNA was ligated with EcoRI/Notl adapters, phosphorylated, and size selected on an agarose gel. Subtraction was done by the previously described procedure (Ausubel et al., 1992b). The cDNA of appressorium-forming conidia was mixed with a 30-fold excess of fragmented cDNA from the nongerminating conidia in a solution containing 50% (v/v) formamide, 10 mM NaPO4, pH 7.0, 1 mM EDTA, pH 8.0, 0.1% SDS, 0.2 mg/mL yeast tRNA, and 5 × SSC (1 × SSC is 0.15 M NaCl, 0.015 sodium citrate), and the mixture was boiled for 5 min and incubated for 24 hr at 37°C. After the hybridization step, the mixture was phenol–chloroform extracted and ethanol precipitated. The DNA pellet was resuspended in water followed by ligation to the EcoRI-cleaved and dephosphorylated λgt11 vector.

cDNA Probe Synthesis and Differential Screening

The poly(A)^+ mRNAs isolated from nongerminating, germinating, and appressorium-forming conidia were used as templates to synthesize first-strand cDNA. Reverse transcription of 1 to 3 μg of poly(A)^+ mRNA was done with α-32P-dATP and Maloney murine leukemia virus reverse transcriptase (RT) using procedures provided by Gibco BRL (Grand Island, NY). The unincorporated α-32P-dATP was removed by a Nensorb-20 column (Du Pont NEN, Boston, MA). The subtracted λgt11 cDNA library was incubated with Escherichia coli Y1090 and then plated on a Luria-Bertani/ampicillin agar plate at a titer of 1000 plaque-forming units per plate. Triplicate nitrocellulose filters were lifted from one plate and hybridized with the cDNA probes synthesized from the nongerminating, germinating, and appressorium-forming poly(A)^+ mRNAs. From each plate, the phage plaques were picked from the area that hybridized with the cDNA probe from the appressorium-forming conidia but not with the other two cDNA probes. After secondary screening, individual plaques that hybridized only with the cDNA from the appressorium-forming conidia were selected. The cDNA inserts were excised with EcoRI, subcloned into the M13mp18 vector, and sequenced (Sanger et al., 1977).

Construction and Screening of a Genomic DNA Library and Sequencing of the cap20 Gene

The genomic DNA was isolated from the mycelia of C. gloeosporioides grown in the mineral medium containing 1% yeast extract and 1% glucose with shaking (200 rpm) for 36 hr (Hankin and Kolattukudy, 1968; Kämper et al., 1994). The genomic DNA was digested with BamHI and subjected to electrophoresis on a 0.7% agarose gel. The gel segment representing DNA fragments within the length of 3 to 7 kb was cut out, and DNA was electroeluted, extracted with phenol, phenol–chloroform, and chloroform, and precipitated with ethanol using standard procedures (Maniatis et al., 1982). The recovered DNA fragments were blunt ended with the Klenow fragment of DNA polymerase I and deoxyribonucleotide triphosphates, ligated with EcoRI/Notl adapters, and ligated...
into the λgt11 vectors. The library was amplified in E. coli Y1090 and screened with cap20 cDNA labeled with α-<sup>32</sup>P-dATP by using a random primed labeling kit (Boehringer Mannheim). The DNA inserts of genomic clones were excised with EcoRI, subcloned into the M13mp18 vector, and sequenced. Nucleotide sequences of both strands were determined by the dideoxy chain termination method of Sanger et al. (1977) using α-<sup>35</sup>S-thiodeoxy ATP. The nucleotide sequence data for the cap20 gene has GenBank accession No. U18061.

DNA Gel Blot Analysis

Genomic DNA was digested to completion with restriction enzymes and subjected to electrophoresis on a 1% agarose gel and transferred to Nytran membranes (Schleicher & Schuell). Prehybridization was at 42°C for 4 to 6 hr in 50% formamide, 5 × SSPE (900 mM NaCl, 5 mM EDTA, 50 mM Na<sub>2</sub>PO<sub>4</sub>, pH 7.4), 5 × Denhardt’s solution (0.1% Ficoll, 0.1% PVP, 0.1% BSA), 0.1% SDS, and 100 μg/ml sheared salmon sperm DNA. The membranes were prehybridized and hybridized as described previously (Maniatis et al., 1982) with 32P-labeled (10<sup>6</sup> to 10<sup>9</sup> pM/μl) full-length cDNA that was 32P-labeled by random primed labeling. After hybridization, the membranes were washed twice for a total of 20 min at room temperature in 2 × SSPE containing 0.1% SDS. An additional wash was performed with 0.2 × SSPE, 0.1% SDS at 65°C for 90 min. The membranes were exposed to x-ray film at −80°C in the presence of an intensifying screen.

RNA Gel Blot Analysis

Total RNA isolated from the fungus was subjected to electrophoresis on a 1% agarose gel containing 2.2 M formaldehyde and blotted onto nitrocellulose membranes as described previously (Maniatis et al., 1982). The conditions for prehybridization, hybridization, and washing were the same as those described for the DNA blot except for the final washing at 65°C for 40 min.

Expression of the CAP20 Protein in E. coli

Polymerase chain reaction (PCR) was used to amplify the cap20 putative open reading frame from cap20 cDNA with a 5' primer containing an Ndel restriction site and 20 nucleotides after the ATG codon and a 3' primer containing a BamHI restriction site and 20 nucleotides before the TAA residues. The fragment was ligated into pET-19b digested with Ndel and BamHI to give the expression plasmid pET-19b(20), which was used to transform E. coli pLysS cells. The recombinant CAP20 protein was induced and purified by a Ni-affinity column using the procedures provided by the manufacturer (Qiagen, Chatsworth, CA). The eluant from the Ni<sup>2+</sup> column was analyzed by SDS-PAGE. The gel from the region of the protein band that appeared at 20 kD was excised. One portion of the gel was directly crushed, and another portion of the gel was subjected to electrophoresion. The eluant was mixed with the crushed pieces for subcutaneous injection into rabbits.

Production of Antiserum against the CAP20 Recombinant Protein and Protein Gel Blot Analysis

To produce rabbit antiserum against the CAP20 protein, the purified CAP20 recombinant protein was injected subcutaneously into rabbits with Freund's adjuvant. Booster injections were administrated every 2 weeks. The rabbit was bled by heart puncture 10 days after the fourth booster, and the serum was decanted after clot formation. For protein gel blot analysis, the nongerminating, germinating, and appressorium-forming conidia were collected from the Petri dishes, broken by vortexing with glass beads for 5 min in 10 mM Tris-buffer, pH 7.0, containing 1% β-mercaptoethanol and 0.5% SDS, and centrifuged at 13,000g for 5 min. After collecting the supernatant, the cell debris was thoroughly mixed with the same buffer and centrifuged. The combined supernatant was concentrated with a Centricon-10 (Amicon, Beverly, MA) apparatus. An aliquot was subjected to SDS-PAGE, blotted onto nitrocellulose membranes, and detected by reaction with anti-CAP20 antiserum using 125I-protein A.

Construction of the Gene Replacement Vector pD20

The BamHI site of pUC18 was deleted by digestion with BamHI, treatment with the Klenow fragment and deoxynucleotide triphosphates, and self-ligation with T4 ligase. The 4-kb genomic DNA fragment containing full-length cap20 cDNA was excised from the λgt11 vector with EcoRI and ligated into the EcoRI site of BamHI-deleted pUC18. The Hpal site at the coding region of cap20 was cut with Hpal, ligated with BamHI linker, and cut with BamHI. The hygromycin gene fused to the Cochliobolus heterostrophus promoter was cut from pBluescript 431 Exp (Bajer et al., 1991) with BamHI and ligated into pUC18 containing the cap20 gene. The final construct is designated as pD20.

Transformation of C. gloeosporioides with the pD20 Vector and Selection of cap20-Disrupted Mutants

C. gloeosporioides protoplasts were prepared as described previously (Bajer et al., 1991). Transformation of protoplasts was done as described previously (Soliday et al., 1989). The protoplasts were then resuspended in STC buffer (1.2 M sorbitol, 10 mM Tris-Cl, pH 7.5, 10 mM CaCl<sub>2</sub>) at various dilutions and plated onto the medium containing 1.2 M sorbitol, 1% yeast extract, 1% glucose, and 2% agar. Overlays of 1% agarose containing 300 μg/mL hygromycin were added 24 hr later. After 7 to 10 days of growth, each of the fastest growing colonies was transferred to the same medium containing hygromycin at 200 μg/mL. After 7 days of growth, each growing transformant was transferred to the same medium containing 200 μg/mL hygromycin. The fastest growing colonies were transferred to avocado potato dextrose agar plate without hygromycin. The genomic DNA of each transformant was isolated as previously described and analyzed by DNA gel blot. Transformants whose cap20 gene was disrupted by pD20 were further analyzed by protein gel blot as described previously to check whether CAP20 was produced.

Pathogenicity Test of cap20 Mutants

The conidia of D4 and D5 mutants and the wild type were obtained from potato dextrose agar supplemented with 1% (w/v) avocado fruit homogenate as previously described. Avocado fruits (Mission Produce Co., Oxnard, CA) harvested from the farm were surface sterilized with 10% bleach for 1 min followed by thorough rinsing with sterilized water. Each fruit was briefly blotted and air dried in the laminar flow hood for 15 min. After being put in a 100% moisture chamber, each fruit was inoculated with 5 drops (2000 conidia per 20 μL per drop) of the wild type or the D4 or D5 mutant within a 1-cm<sup>2</sup> area. The fruits were incubated at room temperature for 6 to 10 days in the high-humidity
chamber. When the fruits inoculated by the wild type showed lesions through the area where the spore suspension was placed, the experiment was terminated. The fruits were longitudinally cut, and the infection symptom was visually examined and photographed. Better Boy tomato fruits were surface sterilized with 70% ethanol and inoculated with 5 drops (2000 conidia per 20 μL per drop) of spore suspension within a 1-cm² area of the fruit surface. The fruits were incubated at room temperature in the high-humidity chamber for 5 to 7 days. When clear infection symptoms were found with the wild type, the experiment was terminated. The surfaces of the fruits were examined and photographed. The thin outer layer of each fruit below the infected surface was excised by freehand, and the infected areas inside the fruit were photographed.

RT-PCR

Ripening tomato fruits were infected with C. gloeosporioides conidia for the indicated periods. The tomato peels or serial thin sections of the fruits below the infected surface were excised by freehand, and the tissue (0.5 to 1.0 g) was added to a 2.0-mL screw-top microcentrifuge tube containing three, 3/16-inch stainless steel ball bearings and 600 μL of TRIzol reagent (Perkin-Elmer Cetus, Norwalk, CT). The tissue was homogenized for 5 min using a Mini-Bead beater (Biospec Products, Bartlesville, OK), and total RNA was isolated according to the manufacturer’s instructions (Perkin-Elmer Cetus). The first-strand cDNA was synthesized using RT in a total volume of 20 μL containing 2 μL of total RNA isolated from the infected tomato, and the cDNA was directly used as the PCR template. Two primers corresponding to CAP20 sequence were synthesized as follows: forward, 5'-ATGTTGACGAATGGCTCAGGTTTAATCGTG-3'; reverse, 5'-CCTATGCCAAGGAATGTG-3'. Thirty cycles of denaturation, annealing, and polymerization were conducted at 92°C for 1 min, at 54°C for 1 min, and at 72°C for 1 min, respectively. The PCR products were analyzed on 3% Nusieve 3:1 agarose gels (FMC BioProducts, Rockland, ME), subcloned in pBlue-script II KS+ (Stratagene), and sequenced (Sanger et al., 1977), using Sequenase (United States Biochemical Corp.) and double-stranded plasmid templates.

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