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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.

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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 4×10^4 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

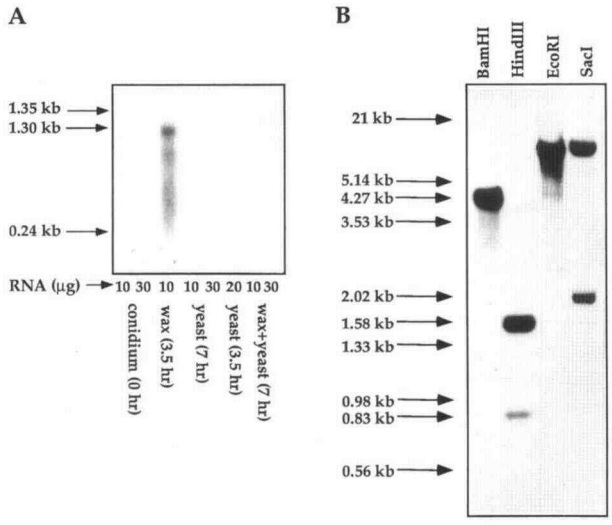

Figure 1. Gel Blot Analyses of the *cap20* cDNA and Gene.

(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 ^{32}P-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 ^{32}P-labeled *cap20* cDNA.

Length markers are indicated at left in kilobases.

isolated from nongerminating, germinating, and appressorium-forming conidia was hybridized with the ^{32}P-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 ^{32}P-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 Ni^{2+} 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

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TGGTAGGGAGCGTAGATGCGTGTCCCGAACCAGGTATGCAATGTACCTCG   -685
GGGGTAGGATACTTGGCCTAAGCCCGCCACCAGTGGCATTCCTGCGCGTC   -635
TCGTTGGTTTTCCTTTCCCTCAGCCTTTGACATGGTCGCGACAAGACGTG   -585
GTGGTGGACGAAAAGCACATGCTAATCGCTCTTGCTCTAGCTCTACCTAG   -535
TCTGCATCCTGACTTCTAAGGGGTTCAAGCAAGCAAGCCTAGACATGCCT   -485
GATCACTTGAACGCAATGGTACTGTCTAGGTAGATAAATACCTTACTTGA   -435
CTTTTTCATTTCTGTCCATGCCCGTCTGTGGCGTCAACCTCGTCACCACC   -385
CATTACATCCTGTCCCATCCCATCCCATCCATCCCGTCCATTGCAGACAA   -335
GCTCGTCTTCTCGTTCCAGCTGTTCCCTCCCTTTTAGCTCTGCCTACTCC   -285
CTCCCACTCACTTCCCTTTCCCCCTCCCATCACCCAAAACCTTCTGCCCG   -235
TCCTTTCCTGTTTCCAAACTCCTCGCGCAACATCTCGTCCGCTCTACACT   -185
CTTGCTTCACCAACCGCCTCTACCTCCATCGTCTACTACTCCTTGACTGT   -135
TGAGCCTCAATCGATCTCAGGTTCCCCTCAACTTTTGCCCTGTACGGTAT    -85
ACCGCTCTCAGCAAACACAATCGCTAGCTTCACGATCTCGAATCTTCCAC    -35
                         ↑
ATCCCAACCCTCGCCCGTTTCCAATCTCGCCTAC ATG TCC AAA ATG    +12
                                   Met Ser Lys Met      4

GCC CAA GTC AAC GGT GAC CTC CCG GCT GTT AAC TCG GCC   +51
Ala Gln Val Asn Gly Asp Leu Pro Ala Val Asn Ser Ala    17

ACT GCT CAGgtaagagtttcgcgcacagacgcaagctttctgttattgg  +100
Thr Ala Gln                                           20

cgctgactccatggcacag CAT CTG CTG GAC ATT CCC GTG ATC   +143
                    His Leu Leu Asp Ile Pro Val Ile    28

CAC GAT GGC GTG GTT GCC TTC AGG AAC AAC CCT CTC GGC   +182
His Asp Gly Val Val Ala Phe Arg Asn Asn Pro Leu Gly     41

AAG AAG TCG ATT GCC ATC GGG GAC TCT GCG TAC CAG ACC   +221
Lys Lys Ser Ile Ala Ile Gly Asp Ser Ala Tyr Gln Thr     54

TTT GCC GCC CCT CTC CTC CCT TAC CTG GCC CGT CCC TGG   +260
Phe Ala Ala Pro Leu Leu Pro Tyr Leu Ala Arg Pro Trp     67

GGC TAC CTG CGC CCT TAC GCG GAA AAG GCC GAC GCT CTT   +299
Gly Tyr Leu Arg Pro Tyr Ala Glu Lys Ala Asp Ala Leu     80

GGC GAC CAG ACC CTG ACC AAG GTC GAG GAG CGC GTC CCC   +338
Gly Asp Gln Thr Leu Thr Lys Val Glu Glu Arg Val Pro     93

GTC ATC AAG AAG CCT ACT GAG GAA CTC TAT GCT GGG GCA   +377
Val Ile Lys Lys Pro Thr Glu Glu Leu Tyr Ala Gly Ala    106

AAA GGC ATC ATC GCC TTG CCG ATT CGT ACC GGC TTT GAG   +416
Lys Gly Ile Ile Ala Leu Pro Ile Arg Thr Gly Phe Glu    119

GCC AAA GAT CAC GTC TTC AAG ACG TAT GCT CAG GAG AAG   +455
Ala Lys Asp His Val Phe Lys Thr Tyr Ala Gln Glu Lys    132

AAG AAG GTT GGC GGC GAG AAC CTG GTG ACC TAC GGC AAG   +494
Lys Lys Val Gly Gly Glu Asn Leu Val Thr Tyr Gly Lys    145

GCC ATC GTC AGT ACT ACT CTC ATC ACT ACT AGC GAA ATC   +533
Ala Ile Val Ser Thr Thr Leu Ile Thr Thr Ser Glu Ile    158

ATC ATC TGG GTT GGA GAT GTC ATG CAC TAC AAG AAG GAG   +572
Ile Ile Trp Val Gly Asp Val Met His Tyr Lys Lys Glu    171

GAG GCC AAG GAC ATT GTG AAC GAG AAG GTC AAC AAC TAA   +611
Glu Ala Lys Asp Ile Val Asn Glu Lys Val Asn Asn STOP   183

GCCTGTAGCTGCACTGGCATTTGGCCCGTTAGTTCCCTTCTCCCCACTTCA  +662
TATACCCTTCTCGTGCGACTCGAATTATCCTTGATTCTTGTATCGCAGTCA  +713
TGACGGCGGACGCTTCAACGACATACAGCAAGGAACGGAATTTATACCTCG  +764
GATCAAGAGCTCTTATTTGTACCTGTCATGTCCTGTTCTCTTATGTTCGTT  +815
TCCTATGTTGGTATATGAGACGGAAGGCTTGCCGTGCGTCGCCCCGCTCCT  +866
CATCACTTGGAACCAACTCTTTGCGGATATATGTCACGATATTGGTGTGTT  +917
CCGCAGAAAATAGGGCGCTTTCCTTGACGACAGCGGAGCTTGCATGGCTTT  +968
CTCCCTGGTCACAGTGATGCGACGGGGCAGCGAGCTTTGCCAGTGCTGGAG +1019
TCGATCATGGAATGAGATGAGCCCAAAATTAATAGATAAGTGTTTCTCATG +1070
ACCTGGCCGCT                                          ↑ +1081
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Figure 2. Nucleotide Sequence and Deduced Amino Acid Sequence of the *cap20* Gene.

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.

and germinated conidia showed very little or no immunologically cross-reacting components. Extracts from appressorium-forming conidia showed a strongly cross-reacting band at 20 kD (Figure 3A). The size of this protein was that expected from the primary translation product of the open reading frame. The time course of expression of the CAP20 protein in the appressorium-forming conidia showed that very little protein could be detected during the first 2 hr of incubation, but a fairly large amount of the CAP20 protein was detected after 4 hr. Even after 28 hr, the protein could still be detected (Figure 3B).

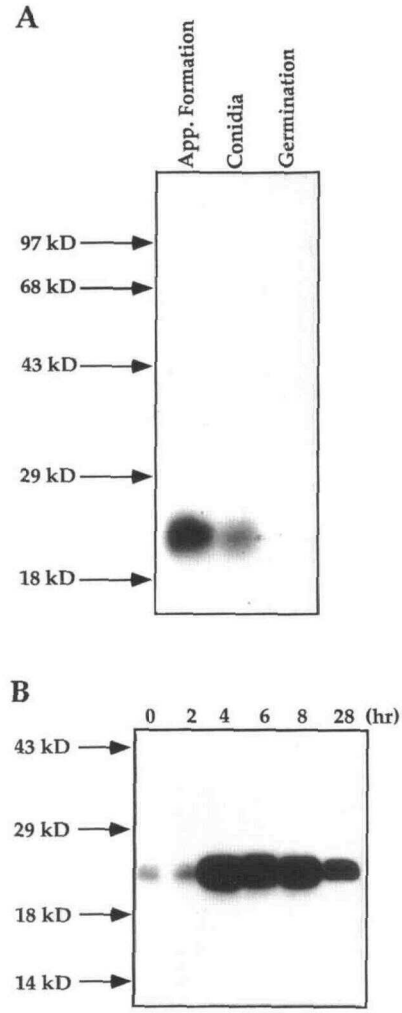

Figure 3. Protein Gel Blot Analyses of Total Proteins from *C. gloeosporioides* Conidia for the CAP20 Protein.

(A) Protein gel blot analyses of total proteins from germinating, nongerminating, 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 ^{125}I-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.

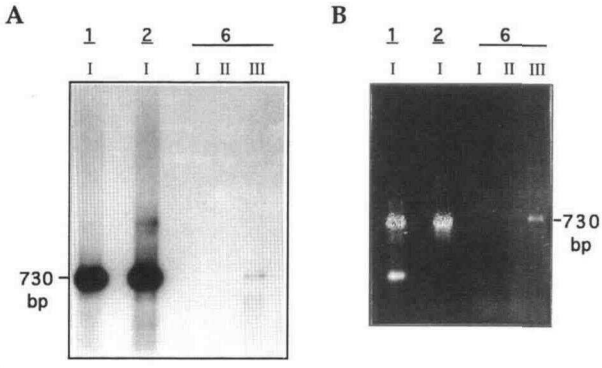

Figure 4. RT-PCR Detection of *cap20* Transcripts in Tomato Fruit Tissue Infected with Wild-Type *C. gloeosporioides.*

(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, at 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 HpaI 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

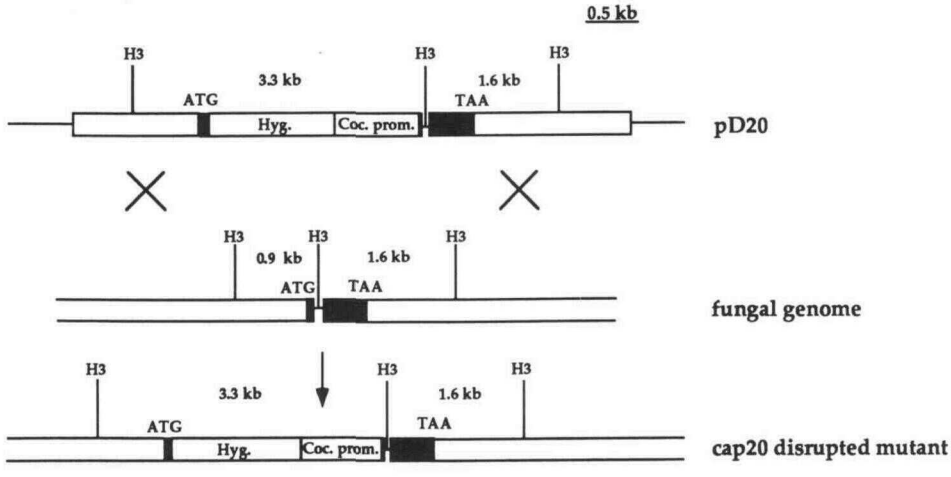

Figure 5. Schematic Presentation of the Strategy Used for *cap20* Gene Disruption in *C. gloeosporioides.*

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.

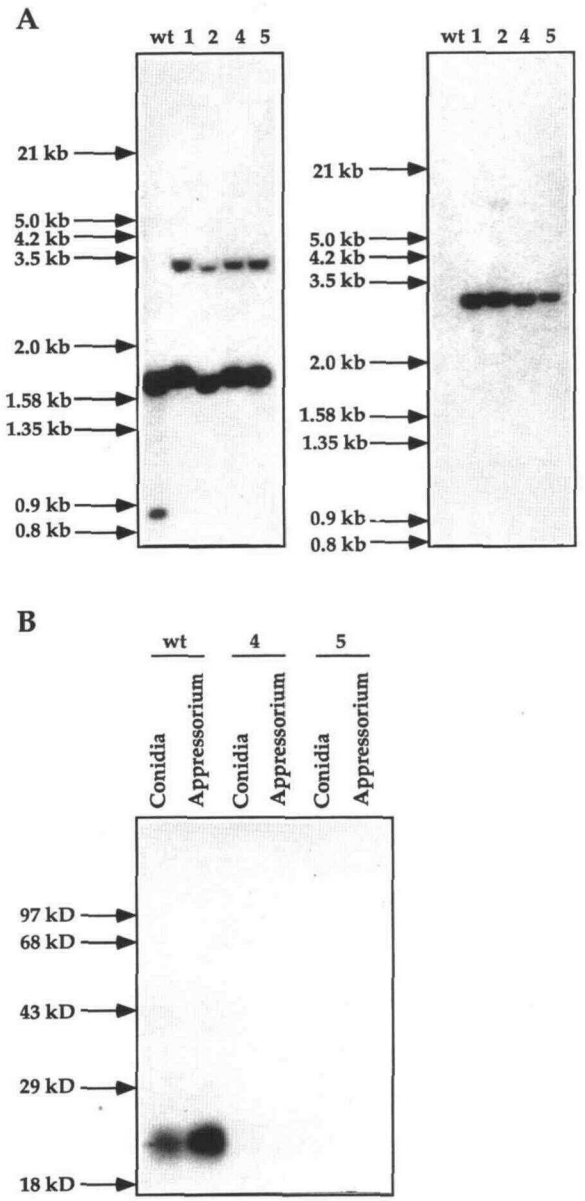

Figure 6. Gel Blot Analyses of *cap20*-Disrupted Mutants of *C. gloeosporioides*.

(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 ^{32}P-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 ^{125}I-protein A for protein detection. The size markers are indicated at left in kilodaltons.

at 0.9 and 1.6 kb, as did the wild type. Four transformants showed hybridization bands at 3.3 and at 1.6 kb, as expected from the insertion of the 2.4-kb hygromycin resistance gene into the 0.9-kb segment (Figure 6A, left). When the hygromycin resistance gene was used as the probe, only the 3.3-kb fragment hybridized, as expected (Figure 6A, right). These mutants, in which the *cap20* gene was disrupted, were examined for their ability to produce the CAP20 protein. Conidia of all four mutants, when treated with avocado surface wax, germinated and formed what appeared to be normal-looking (under the light microscope) appressoria. Immunoblot analysis of the appressorium-forming conidia of two of these mutants (D4 and D5) showed that they did not have immunologically detectable CAP20 protein (Figure 6B). Thus, the gene disruption eliminated the ability of the fungus to produce the protein.

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 symptom, 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.

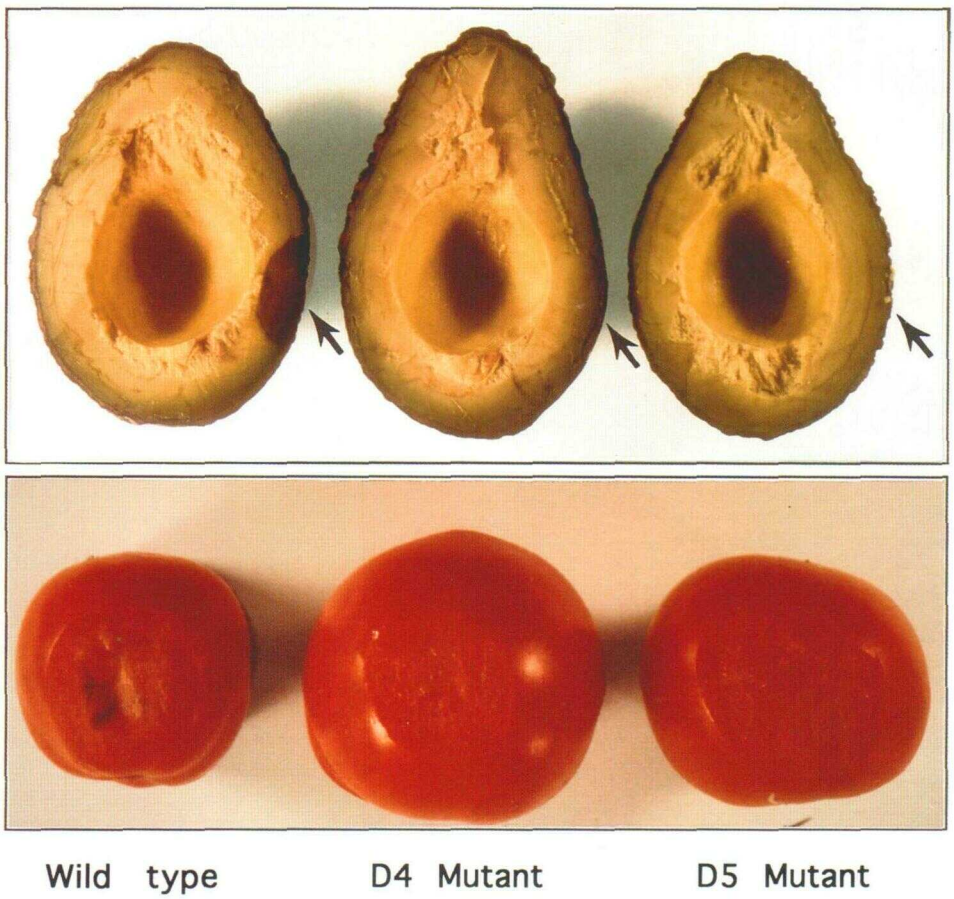

Figure 7. Tests for Pathogenicity of the Wild Type and *cap20*-Disrupted Mutants of *C. gloeosporioides* on Avocado and Tomato Fruits.

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 *mpg1* 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 *mpg1* 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 Rodriquez, 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) avocado fruit homogenate. Conidia were obtained by gently scraping 5- to 7-day-old cultures in Petri dishes flooded with sterilized distilled water. The conidial suspension was filtered through two layers of Miracloth (Calbiochem, San Diego, CA) to remove mycelia, and the conidia were recovered by centrifugation at 5000*g* for 5 min, resuspended in sterilized water, and collected by centrifugation. The number of conidia in the suspension was adjusted to 5×10^7 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 Na_2EDTA, 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 Petri 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 RsaI and AluI to give small blunt-ended fragments. The cDNA synthesized from the appressorium-forming poly(A)$^+$ mRNA was ligated with EcoRI/NotI 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 $NaPO_4^{-2}$, 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-^{32}P-dATP and Maloney murine leukemia virus reverse transcriptase (RT) using procedures provided by GIBCO BRL (Grand Island, NY). The unincorporated α-^{32}P-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 deoxynucleotide triphosphates, ligated with EcoRI/NotI adapters, and ligated

into the λgt11 vectors. The library was amplified in *E. coli* Y1090 and screened with *cap20* cDNA labeled with α-^{32}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-^{35}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 NaH_2PO_4, 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 ^{32}P-labeled (10^8 to 10^9 pm/μg) full-length cDNA that was ^{32}P-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 Nytran 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 NdeI 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 NdeI 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^{2+} 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 electroelution. 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 13,000*g* 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 ^{125}I-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 HpaI site at the coding region of *cap20* was cut with HpaI, ligated with BamHI linker, and cut with BamHI. The hygromycin gene fused to the *Cochliobolus heterostrophus* promoter was cut from pBluescript 431 Exp (Bajar 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 (Bajar 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 $CaCl_2$) 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^2 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-cm^2 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 μg 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′-ATGTCCAAAATGGCCCAAGT-3′; reverse, 5′-CATATATCCGCAAAGAGTTGG-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 pBluescript II KS+ (Stratagene), and sequenced (Sanger et al., 1977), using Sequenase (United States Biochemical Corp.) and double-stranded plasmid templates.

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