CgDN3*: An Essential Pathogenicity Gene of *Colletotrichum gloeosporioides* Necessary to Avert a Hypersensitive-Like Response in the Host *Stylosanthes guianensis

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A gene of *Colletotrichum gloeosporioides* that is induced by nitrogen starvation in axenic culture and is expressed at the early stages of infection of the host *Stylosanthes guianensis* has been identified and its role in pathogenicity tested. The sequence of this gene, named *CgDN3*, indicated that it encodes a protein of 74 amino acids that contains a predicted 18 amino acid signal sequence for secretion of a basic 54 amino acid mature protein with weak homology to an internal region of plant wall-associated receptor kinases. Mutants of *C. gloeosporioides* were produced by homologous recombination in which part of the coding sequence and promoter region of the *CgDN3* gene was replaced with a hygromycin-resistance gene cassette. Mutations in the *CgDN3* gene were confirmed in two independent transformants and Northern (RNA) analysis demonstrated the disrupted *CgDN3* gene was not expressed. The mutants had faster mycelial growth rates in vitro but produced spores that germinated to form appressoria normally on the leaf surface. However, the *CgDN3* mutants were unable to infect and reproduce on intact host leaves. Microscopic analysis revealed small clusters of necrotic host cells at inoculation sites on leaves, suggesting that these mutants elicited a localized, host hypersensitive-like response. The mutants were able to grow necrotrophically and reproduce on leaves when conidia were inoculated directly onto wound sites. The putative promoter region of the *CgDN3* gene was fused to a gene encoding a modified jellyfish green fluorescent protein and introduced into the fungus. Following inoculation, strong expression of green fluorescent protein was observed in primary infection vesicles in infected epidermal cells with weaker expression evident in hyphae growing within infected leaf tissue. These findings indicate that *CgDN3* encodes a novel pathogenicity determinant associated with the biotrophic phase of primary infection

and required to avert a hypersensitive-like response by a compatible host.

The ability of fungal phytopathogens to infect their host depends on their capacity to obtain adequate nutrients while avoiding or negating host defenses. Fungal pathogens that are biotrophs and hemi-biotrophs infect compatible host plants, at least initially, without causing any host cell death (Bailey et al. 1992). Conversely, the rapid death of host cells during the primary stages of an incompatible host-pathogen interaction represents a powerful resistance mechanism in plants (Heath 1998; Maclean et al. 1974) and is known as the hypersensitive response (HR). To establish a successful infection, fungal pathogens must either suppress or avoid triggering a host HR. In many well-characterized, race/cultivar-specific interactions between fungi and plants, it is thought that an HR is determined by an interaction between genes for resistance in the host and genes for virulence in the pathogen (De Wit 1997). In the few highly specific interactions that have been characterized at the molecular level, virulent pathogens can avert triggering an HR if they either lack or have a mutated form of an avirulence gene that corresponds to a specific resistance gene in the host (e.g., Joosten et al. 1994). However, an HR can also be triggered by pathogens attempting to infect incompatible plant species that are not normally hosts to any known races of the pathogen (Heath 1977; Heath and Skalamera 1997). The development of mechanisms to avert triggering nonhost HRs is believed to have been vital for the establishment of basic compatibility between host and pathogen species (Heath 1991). Genes that are essential for fungal pathogens to infect their hosts are beginning to be characterized and have been termed pathogenicity genes. Several classes of these genes have been identified (Oliver and Osbourn 1995; Schäfer 1994). However, fungal pathogenicity genes with a role in negating a potential host HR during the establishment of a compatible interaction have not yet been identified.

The fungal genus *Colletotrichum* includes pathogens that cause disease on a wide range of economically important plant species grown in the tropics (Bailey and Jeger 1992). The ge-

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Nucleotide and/or amino acid sequence data can be found at the GenBank data base as accession no. U94180.

nus *Colletotrichum* includes many pathogens with a hemibiotrophic mode of infection in which host cells that are penetrated remain viable during the early stages of fungal colonization (Bailey et al. 1992). Despite their importance, little is known at the molecular level about the processes used by *Colletotrichum* spp. pathogens to infect their hosts. At the morphological level, spores germinate and form a melanized appressorium on the plant surface to enable direct penetration of the epidermal cell. This is usually followed by the establishment of intracellular vesicles and hyphae in a transient, biotrophic phase followed by further tissue colonization in a necrotrophic phase of infection (Perfect et al. 1999). Methods of gene disruption and complementation of nonpathogenic mutants have been used to identify genes necessary for functional appressorium development (Hwang et al. 1995; Kubo et al. 1996; Perpetua et al. 1994, 1996; Takano et al. 1995) and regulatory processes necessary for primary infection (Dufresne et al. 1998; Yang and Dickman 1999). Presumably, other fungal genes are required for continued parasite development after penetration. Studies of several plant pathogens other than *Colletotrichum* spp. suggest that nutrient deprivation may act as a signal for the expression of genes associated with pathogenesis and virulence (Coleman et al. 1997; Talbot et al. 1993; Van den Ackerveken et al. 1992).

A previous study of gene expression in *C. gloeosporioides* during infection of the tropical pasture legumes *Stylosanthes* spp. suggested that conditions of nitrogen deprivation act as a signal for the induction of some *Colletotrichum* spp. genes at early stages of infection (Stephenson et al. 1997). In the present work we have isolated and characterized a further cDNA of *C. gloeosporioides* that is induced by N starvation in axenic culture. The gene that encodes this cDNA, named *CgDN3*, has no known fungal homologue and is expressed in the fungus at early stages of infection. We show herein that disruption of the *CgDN3* gene leads to a loss of pathogenicity and the production of a hypersensitive-like response in the inoculated host. The *CgDN3* gene therefore represents a novel pathogenicity gene for fungal phytopathogens that may possibly have a role in the establishment of a compatible host-pathogen interaction.

RESULTS

Isolation of a cDNA for a fungal transcript induced by nitrogen starvation in culture and expressed in infected leaves.

A cDNA for *CgDN3* was identified by differential screening of a cDNA library prepared from mycelium of *C. gloeosporioides* that had been subjected to starvation of N compounds (Stephenson et al. 1997). The library was differentially screened with cDNA probes derived from poly (A)⁺ RNA from mycelia subjected to nitrogen starvation, and from poly (A)⁺ RNA of mycelia grown in a complete medium. Seven independent cDNA clones that hybridized preferentially to the cDNA probe derived from mycelia incubated in medium lacking a nitrogen source were selected. Inserts of clones were then used as hybridization probes in Northern (RNA) hybridization to identify cDNAs that represented genes induced by N starvation and were also expressed in leaves of *S. guianensis* infected with *C. gloeosporioides*. One cDNA of approximately 300 bp in length, named DN3-1, hybridized strongly to

an mRNA of approximately 400 nucleotides in length in RNA from fungal mycelia following incubation in media lacking N (Fig. 1). The mRNA detected by the DN3-1 probe was much less abundant in mycelia growing in a nutrient-rich media (Fig. 1). Transcripts hybridizing to the DN3-1 probe were also regulated by carbon starvation although in this instance there was a reduction in transcript abundance, compared with what was observed in the rich growth medium (Fig. 1). The DN3 probe also hybridized to a transcript of similar size in infected leaves at 3 and 4 days after inoculation but no hybridization was detected to transcripts of noninoculated control leaves (Fig. 1). With Northern hybridization methods, the mRNA of DN3 was not detected at early (days 1 and 2; Fig. 1) and late (days 5, 6, and 7; data not shown) stages of infection.

Sequence of *CgDN3* gene and predicted gene product.

A longer cDNA clone (405 bp), named DN3-2, was isolated by re-screening the cDNA library with the DN3-1 cDNA as a hybridization probe. Both cDNAs were fully sequenced and shown to be copies of the same mRNA. The sequence of clone DN3-2 is shown in lowercase in Figure 2. Analysis of this sequence revealed two large open reading frames (ORFs) spanning 208 and 225 bp in frames 2 and 3, respectively. The ORF in frame 3 appeared to encode the gene product as it contained the first ATG sequence on the cDNA and this ORF also had codon usage that matched that of other *C. gloeosporioides* genes in the data bases.

The predicted ORF in frame 3 of the DN3-2 cDNA encoded a protein of 74 amino acids (aa), with a calculated molecular mass of 7.9 kDa and an estimated pI of 9.5. The method of Von Heijne (1983) for predicting cleavage sites of signal sequences suggested that the deduced protein contained a signal peptide of 18 aa at the amino terminus. The predicted 18 aa signal sequence has very significant (>50%) homology with signal sequences involved in the secretion of other eukaryote proteins such as the human activin β -C chain and a surface glycoprotein from the protozoan *Trypanosoma brucei*. Cleav-

Fig. 1. Northern (RNA) hybridization analysis of transcripts of the *CgDN3* gene in the fungus growing in culture and infected leaves. Total RNA was isolated from mycelia after 96 h of growth in rich media (R) and after incubation for the same time in media lacking either nitrogen compounds (N) or carbon compounds (C), as well as from leaves of non-inoculated plants (U) and plants at 1 to 4 days following inoculation. Gel blots were hybridized to the DN3-1 cDNA (*CgDN3*), a DNA probe specific to fungal 25S rRNA, (*Cg rRNA*; Stephenson et al. 1997) and a DNA clone of the entire rRNA repeat of *Aspergillus nidulans* that detects both plant and fungal rRNA (Total rRNA).

age of this signal peptide would leave a mature protein of 56 amino acids with a calculated molecular mass of 5.86 kDa and an estimated pI of 9.32. There were no obvious sequences for retention in the endoplasmic reticulum or targeting to the vacuole and it is most likely that the mature protein is secreted by default. The predicted mature protein was rich in some specific amino acids and contained 11 glycine residues, with valine, proline, lysine, and histidine being the next most abundant (7, 6, 6, and 5 residues, respectively).

Data base searches of GenBank with the gapped BLASTP algorithm indicated that the predicted mature protein had homology (16 aa identities and 13 aa similarities) to amino acid

residues 18–69 of the deduced protein of the *wak3* gene of *Arabidopsis* (He et al. 1999). The *wak3* gene encodes a putative cell wall receptor kinase and the region homologous to the CgDN3 protein is in the predicted extracellular domain of the *wak3* protein, no protein kinase signature domains were present in the CgDN3 protein. A BLASTX search of the coding sequence against the nonredundant GenBank data base revealed a hypothetical protein (accession no. AL161532) of *Arabidopsis* with limited homology (10 aa identities). No fungal homologue was identified in these searches.

Southern hybridization analysis of genomic DNA from biotype B of *C. gloeosporioides* after digestion with *Bam*HI,

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GCAGTGCCACAATGATGTGCGAGATGCGGCCATTGACGGCTTTGGGCCCTAAAGTTTCGCAC 60
TTCTGCACGCTAAGACAATTTTACAAGCCGACATCGGTCAGATGCCTGTCGAAAGGGCCT 120
CTGAAGAGATCAATTCCATCGAGGCGCAGATAGAGCCTCGGTATCCTAGTTAAAGCTCAA 180
CATTCCCAGTTGCGGCCGTCGTCACGAACTAAGATAACGTGAGGTATGGACTCACAGCTC 240
TAAACACTTTCTCTGGAGACCATCGGCCGCTTGTGCATCATCATCCCACTTTCAGTGTATAT 300
GGATGCATTTACGTTCAAAATTCATGCAATCATGAATTGAGTTTGCTCTCTGGGCGGTAG 360
TTCCTGGCTATTCTGGGCGGTATCCCACGAACCACAGCCAGTTAAACTTTTAAATCAATCG 420
CATAGCTGATGACGTATAATACTTTCTACGTAATTAATGACATCCCGGACCGTAGGTA 480
GTTATTGCGTAGTAGTTAAGTACACAAAAGCCCCGCGAGCCTTTTACACGATGTGGTGGTC 540
TTCTGAATAGGCGAATAAGATCACTTGGATAGCTGATCAGTCGGGTAACGCCCGGGTAGC 600
AAAACCTCGTGTAATGATCTGACTTAATACGGTGCTTGCTGGAGCAATGCCATAAGCAG 660
GTGTTGTTGGTTGTTTCGGGGGCTTCAATCAACATCTAAGAATCTTTTAGTGACGCGGTC 720
TAGCCGGCAATGTCAACTCACATGGATCATGCTGTCAGTAACATAGCTCGTCGACGGCCG 780
GTGTTGAAAAGGAATCGCCCCGAAGGCGCATGGTTTCTACTGCGAGTATCCCGGAAAACAA 840
AATACGGACTAAGCGGATTACGGCCAATCTTGGAATGCCCCGAAGCGCCCATGCATGTA 900
TTTGGTTAGCCGTTGGCCAAATTCGCGTTTCAGACGGGAAGCTTACGACTACAAAAGGC 960
TACCATAGTTTAGGGAGACTAGGACACCATAGATTCCCTGACTCAAGTTCAATCCTCGTC 1020
CAAACCACTCTTCGGCGTTCCCCGAGCTTCGACCATGAGTCATGGTCGTATGTAGGTCCC 1080
AATGTACCCGCGGATTGACTGTGTATAGTAGTGTGAATTGCGTCTAGCCGTAAATCAT 1140
TGGTTCGGAGAGAACCGCTGGCCACAATCCTTATGAATCGCATGATAAAGCGTAGGTACT 1200
GTCAAAAGCATATTCTCAATGTGTTAACTCCCGATGGTAACCTCCGATGCGATGAGGCCT 1260
TGCAGTCGTGGATAGAGCGTGCGATGACCGCTCTTGTCTAGCTTGACAGATGAGCAATATA 1320
AGGAGAAGCGAGTTTCCTCTGCACCAGCGAGGTCGAACGAACAGGCCGCACTCATCTGCC 1380
aattcttccacagataagaccatcaataaacgtccattccatacaaaagaaaaaactact 1440
                                     M   Y   2
cccgaagcctcctctttttcctcctcgctcggttcccttcaccgctgcccgttccctgtcgccg 1500
S   R   S   L   L   F   F   L   L   V   V   P   F   T   A   A   ★   V   P   V   A   22
aacagaagcacaaacggcaagccgatacagTATGTGCGCCTCTCCTCTCTCTATTGAACCT 1560
E   Q   K   H   N   G   K   P   I   H   30
AGACCATTGTATTTCATCTCGACCCCAATAATATTTTCGCTTGCCGTCAACAAGTAGAGCA 1620
TCGCACATCTATTAAACATTGCTCCAAGtgaaagtctcctggcatgggcacgggcacctatt 1680
                                     V   K   V   P   G   M   G   T   G   T   Y   43
atccgggagaccatggacatgatggcaatgtagccgtatacaatcttgcgccactatggca 1740
Y   P   G   D   H   G   H   D   G   V   V   A   V   Y   N   L   A   H   Y   G   63
atgtaaacggccctaagaaaggggggaaaccgtaaataggaaagaccatcatcagatggg 1800
N   V   N   G   P   K   K   G   G   K   P   *   74
gtcacgactgggctgcagttctacagactaaatctgaagtcgccaatataaacacagagaag 1860
ataacagacaaaagaaatagttttcgcatg 1890

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Fig. 2. Full nucleotide and amino acid sequence of the *CgDN3* gene from *Colletotrichum gloeosporioides*. Translation start is in bold and shaded; deduced amino acid sequence is shown; position of deduced cleavage site for the putative N-terminal signal sequence is indicated by a star. Nucleotide sequence was determined from overlapping genomic clones; sequence of the cDNA clone pDN3-2 is in lowercase. Putative TATA and CAAT boxes in the promoter region are boxed; putative *cis* elements involved in gene regulation (Blinder and Magasanik 1995; Chiang et al. 1994; Fu et al. 1995) by nitrogen supply are underlined and shaded. A near-consensus putative transcription start region and associated upstream pyrimidine-rich motif typical of many fungal genes (Balance 1986) are underlined. This sequence can be found at the GenBank data base as accession no. U94180.

EcoRI, *EcoRV*, or *HindIII*, gave a single band when hybridized to the DN3-1 cDNA probe, consistent with this being a single-copy gene (results not shown). The gene, termed *CgDN3*, corresponding to the DN3 cDNAs was cloned and a gene sequence assembled from two overlapping genomic DNA sequences. The gene sequence (Fig. 2) indicated the presence of a small intron in the coding region (nucleotides 1525–1638).

The genomic region upstream of the cDNA sequence was analyzed with the TESS algorithm to identify putative transcriptional elements. Putative TATA and CAAT boxes were identified at positions 1317–1320 and 1227–1230, respectively (Fig. 2). The promoter sequence upstream of the putative TATA box contained motifs (Fig. 2) that in *Neurospora crassa* have been shown to bind products of the nitrogen regulatory genes *nit-2* and *nit-4*.

The *CgDN3* gene is expressed early after inoculation in fungal infection vesicles and hyphae.

The amount of fungal biomass present in infected leaves is very low until 3 days after inoculation when it increases rapidly (Stephenson et al. 1997). The increase in fungal biomass can be observed in Northern hybridization experiments by measuring fungal 25S rRNA with a fungal-specific hybridization probe (Fig. 1). Northern blotting lacks the sensitivity necessary to study fungal mRNAs and rRNAs at early stages of infection (Fig. 1). Consequently, reverse transcriptase-polymerase chain reaction (RT-PCR) was used to determine whether *CgDN3* transcripts were expressed in the fungus at an early stage after inoculation (Fig. 3). The results demonstrated the presence of *CgDN3* transcripts in leaves at 1 day after inoculation (Fig. 3). Similar experiments failed to detect *CgDN3* transcripts in RNA of spores used for inoculation (data not shown). The RT-PCR product detected in inoculated leaves was estimated by gel electrophoresis as 220 bp in size and this corresponded to the 222-bp PCR product size predicted from the cDNA sequence. The use of genomic DNA as a template in a PCR with the same primers led to a product approximately 340 bp in size because of the intron, and this was easily distinguished from the RT-PCR product. The absence of any 340-bp product in the RT-PCRs demonstrated that the RNA templates used for RT-PCR analysis were free of genomic DNA contamination. Subsequent analysis, with quantitative, real-time RT-PCR (Perkin-Elmer, Foster City, CA), showed that *CgDN3* transcripts, relative to fungal 25S rRNA, were as abundant at 1 day as at 4 days after inoculation

Fig. 3. Detection of transcripts of the *CgDN3* gene at an early infection stage by reverse-transcriptase polymerase chain reaction. Total RNA was isolated from N-starved mycelium in culture (N), leaves of non-inoculated plants (U) and of plants at 1 and 4 days following inoculation. Purified genomic fungal DNA (G) and the cDNA clone DN3-2 (cD) were used as control templates and a reaction lacking added template (NT) was used to test for contamination.

and were approximately three times more abundant than those in fungal mycelium subjected to nitrogen starvation in culture (data not shown).

Experiments were designed with promoter-reporter constructs to assess where expression of the *CgDN3* gene may be localized during infection. A reporter gene encoding a jellyfish green fluorescent protein (GFP) was selected, as this permits direct observation of gene expression. The GFP expression patterns in a fungal transformant (MD12) that contained a *CgDN3* promoter-GFP gene were compared with those observed in a transformant (M7) that contained a gene that comprised the strong constitutively expressed promoter of the glyceraldehyde-3-phosphate dehydrogenase (*gpdh*) gene of *Aspergillus nidulans* linked to a GFP coding region. The latter acted as a positive control for the detection of GFP in fungal structures in infected leaves. In this M7 positive control, GFP expression was observed by light microscopy in spores and also in appressoria formed both on glass slides and on the leaf surface (Fig. 4). With light microscopy it was also possible to see expression of GFP in superficial hyphae, and what appeared to be subcuticular and other hyphae growing within infected plant tissue (Fig. 4). GFP was also clearly present in surface mycelia of M7 that erupted from necrotic areas forming fungal acervuli (not shown).

In axenic cultures of transformant MD12 that contained the *CgDN3* promoter-GFP construct, expression of GFP was observed in hyphae by light microscopy after transfer to media lacking nitrogen-containing nutrients (data not shown). Expression of GFP was not observed in conidia and was also not observed in appressoria formed on either glass slides or on the leaf surface. With light microscopy, expression of GFP was observed in infection vesicle structures (Fig. 4), apparently within the host epidermal cell or cell wall at a lower focal plane beneath appressoria. In contrast to the M7-positive control, there was no expression of GFP in hyphae of MD12 that emerged from leaves at later stages of infection. No GFP was observed with light microscopy in hyphae of MD12 growing within leaf tissue. To provide greater resolution, confocal microscopy was used to provide scanned optical sections of infection sites of leaves following inoculation with MD12. An example is presented in Figure 4 as an integrated image showing all fluorescent structures. Confocal images confirmed that strong GFP expression was present in vesicles at penetration sites but also revealed weak GFP expression in fungal hyphae that appeared to be connected to the vesicle structures. In some instances, hyphae appeared to be growing at a plane just below the leaf surface, and these may have been subcuticular hyphae (Fig. 4).

Targeted disruption of the *CgDN3* gene by homologous recombination.

To generate mutations in the *CgDN3* gene, a disruption vector (p3Phph) was constructed to replace a part of the *CgDN3* gene with a chimeric hygromycin resistance (*hph*) gene via transformation and homologous recombination (Fig. 5). The *hph* gene cassette included the promoter and terminator sequences of the *TrpC* gene of *Aspergillus nidulans*. This cassette was flanked by *CgDN3* sequences at positions 1–774 and 1446–1781, as shown in Figure 5. A double recombination event between homologous regions of the p3Phph vector and the *CgDN3* gene would be expected to replace 675 nu-

cleotides of the *CgDN3* gene with the *hph* gene. The sequences deleted from the *CgDN3* gene would include the putative transcriptional start and the TATA and CAAT transcriptional elements followed by the first four codons including the translational start. This would be predicted to prevent both transcription and translation of the *CgDN3* gene.

Protoplasts of *C. gloeosporioides* were transformed with p3Phph and several putative transformants were selected after plating on a medium containing hygromycin. The predicted gene replacement event should result in a 1.4-kb increase in the size of bands hybridizing to a *CgDN3* probe in Southern blots with restriction enzymes that cut outside the *CgDN3* gene and *hph* gene sequences. To test whether the predicted gene replacement event had occurred, genomic DNA was extracted from two independent transformants and digested with

*Sma*I, which has no site within *CgDN3*, and the *hph* gene cassette. Southern hybridization analysis was undertaken with DNA probes derived from both the *hph* gene and the insert of the DN3-2 cDNA (Fig. 6). The DN3-2 probe hybridized to a band of approximately 13 to 14 kb in the wild-type genotype but to a larger band of approximately 15 kb in both of the transformants. The *hph* probe hybridized to a band of identical size to that detected by the *CgDN3* probe in the transformants but failed to hybridize to the DNA of the wild-type genotype. These observations (Fig. 6) indicated that homologous recombination had occurred, as predicted in Figure 5, and that, in each of the transformants, the *hph* gene had replaced part of the *CgDN3* gene. As only two transformants were examined and both were gene replacement events, it appears the frequency of homologous recombination events was probably high.

Fig. 4. The *CgDN3* promoter drives expression of a green fluorescent protein (GFP) reporter gene in infection vesicles and infection-related hyphae. **A** and **B**, Light micrographs of infection sites of leaves inoculated with transformant M7 expressing a GFP gene driven by the strong constitutive *gpdh* promoter of *Aspergillus nidulans* showing expression in conidia (c), appressoria (a), superficial hyphae (h), and subcuticular hyphae (s). **A**, Inset shows expression in a spore and appressorium of M7 formed in vitro. **C**, Light micrograph of an infection site on leaves inoculated with transformant MD12 expressing GFP driven by the *CgDN3* promoter. No expression was observed in conidia and appressoria but GFP expression was detected in vesicle structures (v) in host epidermal cells. **D**, Integrated image of multiple, confocal, optical sections through a leaf following inoculation with transformant MD12. Strong GFP expression can be seen in vesicle structures with weaker expression in subcuticular (s) and other hyphae in the plant tissue. The photomicrographs were all taken at 72 h following inoculation. **A**, **C**, and **D**, Bar = 10 μ m. **B**, Bar = 20 μ m.

An experiment was undertaken to test whether transcription of the *CgDN3* gene had been abolished in the hygromycin-resistant recombinants described above. The wild-type genotype UQ62 and one of the putative *CgDN3* mutants were grown in rich medium for 4 days and then incubated in a medium lacking nitrogen compounds for a further 4 days. Total RNA was extracted and Northern hybridization analysis carried out with the DN3-2 cDNA probe (data submitted but not shown). As expected, this revealed a strongly expressed *CgDN3* transcript in the wild type, but no hybridization to any transcript homologous to the *CgDN3* gene was detected in the transformant. The Southern and Northern blot analyses indicated that the strategy used to disrupt the *CgDN3* gene was successful. The two selected transformants were then termed *CgDN3* mutants 1 and 2.

Disruption of the *CgDN3* gene affects hyphal extension rates and abolishes pathogenicity.

Radial growth rate measurements on solid V8 juice agar plates indicated that the *CgDN3* disruption mutants had enhanced hyphal extension rates, compared with wild-type cultures (Table 1). Two transformants obtained with vectors carrying selectable marker and reporter genes only were also tested to determine whether the transformation process non-specifically affected radial growth rates, but these were identical to those of wild type (data not shown). The cultures of the *CgDN3* mutants sporulated normally and produced conidia of normal morphology and dimensions. When these conidia were applied to glass slides in droplets of water and/or potato dextrose media they adhered and germinated, producing melanized appressoria that could not be distinguished in morphology from those of the wild type. However, there appeared to be a 32 to 40% reduction in the rate of appressorium formation in the mutants once the spores had germinated in vitro (Table 1). When appressoria were not formed upon germination the germ tubes from spores of the *CgDN3* mutants in vi-

tro were more elongated than those of the wild type, which is consistent with the extended hyphal extension rate detected in radial growth assays (Table 1). However, when appressoria were formed there was no difference in germ tube length. On the leaf surface, there were no significant differences in the rates of appressorium formation of the *CgDN3* mutants with those of the wild type. In addition, germ tube length was highly variable on the leaf surface but was not significantly different from the wild type in either of the mutants (Table 1).

The two *CgDN3* mutants were used to inoculate 6-week-old plants of *Stylosanthes guianensis* cv. Graham, a cultivar fully susceptible to the race 3 wild-type isolate of *C. gloeosporioides*. The ability of the *CgDN3* mutants to infect host leaves was compared with that of the wild-type isolate UQ62 and a control transformant obtained with a transformation vector lacking any sequences of *C. gloeosporioides*. Five to 6 days after inoculation of host leaves with droplets of spore suspension, typical disease symptoms were produced by the wild type and control transformant, with large, clearly visible, necrotic lesions (>5 mm in diameter) and fungal acervuli developing at the sites of inoculation droplets (Figs. 7 and 8). After 7 to 10 days, the leaves inoculated with either the wild type or control transformant were blighted, and some dropped from the plant (Fig. 7). In contrast, plants inoculated with the *CgDN3* mutants remained green, continued to grow normally, and were producing new leaves by 7 days (Fig. 7). Following inoculation of the leaves with conidia of the *CgDN3* mutants, small (<1 mm in diameter), brown, necrotic flecks were visible where the droplets of spore suspension had been applied, but no lesions or fungal acervuli developed (Fig. 8). Quantitative analysis of disease ratings in replicated experiments is shown in Table 1, and large and highly significant ($P < 0.01$) differences were observed between the wild type and the *CgDN3* mutants on intact leaves. Examination of leaves inoculated with the *CgDN3* mutants by light microscopy

Fig. 5. Diagrammatic representation of homologous recombination events associated with replacement of part of the *CgDN3* gene with the hygromycin resistance (*hph*) gene cassette by the disruption vector p3Phph, which contained two genomic sequences (positions 1–744 and 1446–1781, shown in Figure 2) derived from the *CgDN3* gene separated by a heterologous *hph* gene cassette. Upstream genomic sequence of the *CgDN3* gene consisted of a promoter region; downstream region, part of the transcribed region. The *hph* gene cassette comprised the promoter (P) and terminator (T) of the TrpC gene of *Aspergillus nidulans* with the coding region of the *hph* gene of *Escherichia coli* (HygR). Position of the probe (cDNA clone DN3) used in Southern hybridization experiments (Fig. 6) is shown.

showed that the flecks on leaves inoculated with the *CgDN3* mutants consisted of small clusters of necrotic host cells with dark pigmentation (Fig. 8). Appressoria were visible in association with the necrotic cells and most of these necrotic cell clusters comprised stomatal guard cells and stomatal subsidiary cell complexes (Fig. 8). Although some superficial fungal hyphae were evident growing on the surface of the leaves inoculated with the *CgDN3* mutants there was no evidence of hyphal growth or sporulation within the host tissue following inoculation with these mutants.

Although the mutants were unable to infect intact leaves, they were able to colonize wound sites on leaves. When conidial suspensions of the mutants were applied to leaf tissue that had been gently crushed by the tips of forceps, leaf necrosis and fungal acervuli were visible about 3 to 4 days after inoculation, and leaf blighting and leaf loss occurred shortly thereafter (Fig. 7). Similar symptoms were observed in wounded leaves inoculated with wild-type conidia (Fig. 7). Inoculation of pre-wounded leaves with the *CgDN3* mutants led to disease ratings that were very similar to those produced by the wild type (Table 1). No disease symptoms were observed in wounded, mock-inoculated controls (Fig. 7).

Fig. 6. Southern hybridization analysis of *CgDN3* sequences in genomic DNA from biotype B of *Colletotrichum gloeosporioides* (wt) and from two putative *CgDN3* mutants (mt1 and mt2) derived by homologous recombination with vector p3Phph. DNA was digested with *Sma*I and hybridized with the hygromycin resistance (*hph*) gene cassette (lanes marked *hph*), then the probe was removed and the blot probed again with the pDN3-2 cDNA insert (lanes marked *CgDN3*). Sizes of *Hind*III-digested λ standards (λ) are shown in kb.

DISCUSSION

The results of experiments described herein demonstrate that the *CgDN3* gene of *C. gloeosporioides* is essential for pathogenicity on intact leaves of *S. guianensis*. The principal evidence for this proposition was obtained by targeted gene disruption to produce mutants with a specifically inactivated *CgDN3* gene. These mutants were unable to produce functional transcripts of the *CgDN3* gene and were nonpathogenic when conidia were inoculated on intact leaves even though the conidia germinated and formed appressoria on the leaf surface. The deduced amino acid sequence of the *CgDN3* gene product has no fungal homologue in the public data bases. Therefore, the *CgDN3* gene appears to encode a novel pathogenicity determinant in phytopathogenic fungi.

Leaves inoculated with the *CgDN3* mutants had small, brown flecks and microscopic analysis indicated that these were the result of the necrosis of small clusters of cells associated with fungal appressoria. The stomata and stomatal subsidiary cells were usually affected; these cells are the preferred sites of penetration of *C. gloeosporioides* on *Stylosanthes* spp. (Ogle et al. 1990). These results suggest that the *CgDN3* mutants elicited a hypersensitive-like response on what is normally a compatible host. A host hypersensitive-like response with dark brown pigmentation like that described herein was not observed previously in race-specific resistance reactions of *S. guianensis* to incompatible races of *C. gloeosporioides* (Ogle et al. 1990). As a working hypothesis, therefore, we propose that the *CgDN3* gene is essential to avoid eliciting what we term a "cryptic" hypersensitive-like response that may be part of a general, nonhost, defense reaction. It has been hypothesized that host cell death is the default reaction during penetration by microbes and that the evolution of mechanisms that negate this reaction is necessary to establish basic compatibility with the host (Heath 1991; Heath and Skalamera 1997).

The *CgDN3* mutants were able to grow necrotrophically on host leaves and form spreading necrotic lesions when conidia were applied to wound sites. This suggests that *CgDN3* is not essential for the necrotrophic phase of pathogen development in the host. In support of this notion, Northern blot analysis failed to detect *CgDN3* transcripts at stages of infection greater than 4 days following inoculation and the *CgDN3* promoter did not drive detectable levels of GFP reporter in fungal hyphae emerging from infected leaves at late necrotic

Table 1. Effect of disruption of the *CgDN3* gene on hyphal growth rate, germination, and appressorium (App) formation both in vitro and on intact leaves and on the infection index on intact and on prewounded leaves^a

Fungal strain	Germination and App formation in vitro							Germination and appressorium (App) formation on leaves			Infection index	
	Radial growth rate (mm/day)		App / germling (%)		Germ tube length (μm) in PDA						Wounded plants	Intact plants
	PDA media	V8 media	H ₂ O	PDA	-App	+App		App / spore (%)	App / germ-ling (%)	Germ tube length to App (μm)		
UQ62	7.6 ± 0.9	7.3 ± 0.9	78.9	75	78 ± 41	49 ± 22		85	95.8	14.7 ± 10.3	6.7 ± 0.6	6 ± 1.1
DN3 1	11.5 ± 1.5	15.2 ± 1.0	53.7	45.4	187 ± 59	43 ± 11		91	97.4	5.4 ± 3.0	7 ± 1.7	0.7 ± 0.6
DN3 2	12.7 ± 1.6	15.3 ± 0.9	49.4	48.6	121 ± 28	54 ± 14		77	96.4	27.2 ± 13.9	7 ± 1.1	0.3 ± 0.6

^a Two mutants (DN3 1 and 2) were obtained from the wild type (UQ62) by targeted gene disruption of the *CgDN3* gene. Radial growth rates were obtained on solid V8 or potato dextrose agar (PDA) media in petri dishes. Formation of appressoria and germ tube lengths was assessed by light microscopy following germination in either water or one-quarter strength-potato dextrose media on glass slides or on inoculated leaf surfaces. Infection indices were estimated at 10 days after inoculation with the key of Chakraborty (1990). Means and standard deviations are shown for at least three independent experiments.

stages of infection. Because the conidia of *CgDN3* mutants were able to germinate and form appressoria on intact leaves but were unable to establish a compatible interaction, it seems probable that the *CgDN3* gene is important in the primary infection processes at penetration or early in the post-penetration phase.

The cytology of primary infection processes in the *S. guianensis*-*C. gloeosporioides* interaction has generally been difficult to study. This is primarily because of the low frequency (approximately 5%) of successful penetration events from appressoria and the poor resolution of infection structures in

leaves of this host species (Munaut and Maraite 1998; Ogle et al. 1990). Generally, it would appear that following penetration of the host cuticle and cell wall, a spherical fungal vesicle is formed either in the host cell wall (Vinijsanun et al. 1987) or more usually in the epidermal cell itself (Ogle et al. 1990). From the vesicle, a range of fungal structures can develop. These include intracellular, intercellular, superficial, and subcuticular hyphae (Irwin et al. 1984; Ogle et al. 1990; Vinijsanun et al. 1987). The subcuticular hyphae appear to grow between the cuticle and epidermal cells and initiate infection in epidermal cells remote from the primary infection site. In-

Fig. 7. Effect of disruption of the *CgDN3* gene on development of macroscopic disease symptoms following inoculation of leaves of *Stylosanthus guianensis* cv. Graham with *Colletotrichum gloeosporioides*. Plants were either (A and D) mock inoculated with water, (B and E) inoculated with a conidial suspension of a *CgDN3* mutant, or (C and F) inoculated with conidia of the wild-type fungus. Leaves were either (A–C) untreated or (D–F) wounded by gentle squeezing with forceps prior to application of the inoculum. Photographs were taken 10 days after inoculation; wound sites (w) and blight symptoms in compatible interactions (b) are arrowed. C, E and F, Mock-inoculated wound site (w) and blighted leaves (b) are arrowed.

Fig. 8. Microscopic features of *Stylosanthes guianensis* at 7 days following inoculation of intact (nonwounded) leaves with conidial suspensions of either (A) the wild-type isolate of *C. gloeosporioides* or (B–D) a *CgDN3* mutant. A, Spreading lesion perimeter (l) and fungal acervuli (a) in the compatible interaction. B, Small hypersensitive necrotic flecks (h), associated with inoculation droplets with *CgDN3* mutants within the perimeter (p) of the inoculum drop. C and D, Higher resolution examination of these hypersensitive flecks, showing necrotic host cell clusters (c), necrotic stomatal cells (st), and subsidiary cells (sb). Superficial fungal hyphae growing on the leaf surface (sh) and fungal spores (s) are also shown. A and B, Bar = 1 mm, C and D, Bar = 100 and 10 μ m, respectively.

tercellular hyphae, on the other hand, colonize the leaf photosynthetic cells and cause host cell necrosis that becomes visible after 4 days following inoculation (Ogle et al. 1990; Vinijsanun et al. 1987). Transcripts of the *CgDN3* gene were detected in the fungus on the plant at 24 h after inoculation, which coincides with the putative biotrophic stage of infection (Ogle et al. 1990; Sharp et al. 1990; Vinijsanun et al. 1987). The *CgDN3* promoter region was not active in spores, germ tubes, or appressoria, but appeared to direct strong expression of a GFP reporter gene in primary infection vesicles and weak expression in subcuticular and other infection-related hyphae formed post-penetration. Strong expression of the promoter in the infection vesicle is consistent with the gene being associated with biotrophic stages of fungal development. The expression pattern of the *CgDN3* gene in infection vesicles in the fungus described herein, taken together with the observation of a strong hypersensitive-like response following inoculation with the *CgDN3* mutants, suggests that this gene may play a role in maintaining biotrophy during the early stages of infection.

The mechanism by which the CgDN3 protein interacts with the host during infection was not elucidated. However, sequence analysis suggests that the *CgDN3* gene product is most probably a secreted peptide that potentially may diffuse to the surface of the host plasma membrane and thus may act as a molecular suppressor of the hypersensitive-like response. The presence of diffusible pathogen compatibility factors that can suppress host defense responses have been described previously in other host-fungus interactions (Oku et al. 1987; Shiraishi et al. 1992; Walton 1996). The predicted CgDN3 mature protein product showed some homology to the extracellular domain of a family of plant cell wall receptor protein kinases (He et al. 1999). The function of this family of plant protein kinases is not known. It has been hypothesized that the extracellular domain is involved in signal recognition, and the observation that some members of the family are inducible by plant defense regulators such as salicylic acid has suggested a role for these molecules in plant-pathogen interactions. An alternative hypothesis is that disruption of the *CgDN3* gene leads to an alteration in a tightly programmed developmental process that is necessary to establish a hemi-biotrophic infection. It appears from our analysis that the *CgDN3* gene may have a role in regulating or restricting hyphal extension rates (Table 1). However, no substantial differences were observed in the development of leaf surface infection structures in the *CgDN3* mutants and that of the wild type. More detailed analysis of infection time courses and penetration events will be necessary to determine if there are subtle alterations in the timing of development of intracellular infection structures.

The approach used for the identification of the *CgDN3* gene described herein was based on the hypothesis that nitrogen starvation may act as a cue for the induction of fungal genes involved in pathogenesis. This has now been demonstrated to be the case for genes implicated in pathogenicity and virulence in other fungal plant pathogens (Coleman et al. 1997 and references cited therein). Within the putative promoter of the *CgDN3* gene there are motifs found in the promoters of other fungal genes that are induced by nitrogen starvation (Fu et al. 1995). It is therefore possible that signal pathways originally evolved for the regulation of nutrient utilization in saprophytic growth may also regulate the expression of patho-

genic genes necessary for parasitic growth. To test this hypothesis, it should now be possible to identify the regulatory elements necessary for expression of the *CgDN3* gene in infection structures and under nitrogen starvation.

MATERIALS AND METHODS

Fungal isolates, culture conditions, microscopic analysis, and pathogenicity tests.

Isolate UQ62 of biotype B of *C. gloeosporioides* (Manners et al. 1992) was used for this study and has been described extensively before (He et al. 1996, 1998; Masel et al. 1993; 1996; Sharp et al. 1990). This culture can be accessed as reference no. 90710 at the American Type Culture Collection (Manassas, VA). Culture conditions and media for maintenance of this strain have been described previously (Stephenson et al. 1997). The rich media used for growth of the fungus were Czapek's salts, 10 mM trisodium citrate, 2% sucrose, and 1% Casamino Acids (Difco, Detroit, MI). Procedures for nitrogen starvation experiments were as described by Stephenson et al. (1997) and involved growing mycelium in rich media with a subsequent transfer to fresh rich media without nitrogen-containing salts and the Casamino Acids.

Pathogenicity tests of wild-type and transformed fungal genotypes were undertaken in contained conditions as described by Masel et al. (1996) and were conducted on three independent occasions with at least two plants per treatment. For wound treatments the leaves were crushed gently with fine forceps before application of conidial suspensions (10^6 spores per ml). Disease ratings were assessed as described by Chakraborty (1990).

For microscopic analysis of spore germination and appressorium formation, the fungus was grown on V8 and one-quarter strength potato dextrose agar media. Spores were collected from the cultures by flooding the plates with sterile water, scraping the plates, and filtering the spore suspension. The inoculum (10^6 spores per ml) was sprayed on leaves of *S. guianensis* cv. Graham until runoff, plants were placed in the dark in a dew chamber at 24°C for 48 h, and then three leaves were sampled. Whole leaves were cleared in 75% ethanol, rehydrated, and then submerged in 0.1% Calcofluor White BT (Sigma Aldrich, St. Louis, MO) in 0.1 M Tris (Sigma Aldrich), pH 8.3 solution, and stained for 5 min. Leaves were then washed three times in distilled water and analyzed by fluorescence microscopy. For the analysis of infection structures formed in vitro, drops of spore suspension in either water or one-quarter strength potato dextrose media were placed on slides, spread on the surface, and left for 30 min for the spores to attach to the slide. The excess was washed with H₂O or one-quarter strength potato dextrose media. Slides prepared this way were then placed in petri dishes with filter paper soaked in water to maintain the humidity, sealed with Parafilm, and placed in a dark cabinet for 24 h under UV light. For detection, a drop of Keane staining solution (Keane et al. 1988) was applied, the slides were washed in water, and germinated spores were observed directly by light microscopy.

cDNA library construction and differential screening.

A λ gt10 cDNA library was prepared with mRNA extracted from mycelia grown in rich media in axenic culture and then transferred to media lacking all nitrogen compounds, as de-

scribed by Stephenson et al. (1997). Duplicate blots of approximately 5,000 plaques from the cDNA library were differentially screened with first-strand, ^{32}P -labeled cDNA prepared with poly(A)⁺ RNA isolated either from mycelium grown in rich medium or from mycelium grown in the same media but then transferred to a medium of the same basic composition but lacking all nitrogen compounds. Clones that hybridized preferentially to the cDNA probe from mycelia incubated in the nitrogen-free medium were selected for further analysis.

Isolation and characterization of cDNA and genomic clones.

Clones that hybridized preferentially to the cDNA probe from mycelia in D-N medium included λ DN3-1. The insert of λ DN3-1 was amplified from λ gt10 by PCR, subcloned into pGEM-T (Promega, Madison, WI), and was named DN3-1. The DN3-1 insert was then used to rescreen the library and a longer clone, named DN3-2, was subcloned into pGEM-T. The inserts of both DN3-1 and DN3-2 cDNAs were sequenced fully in both directions with an automated 373A DNA Sequencer (Applied Biosystems, Pasadena, CA) and the ABI PRISM DyeDeoxy Terminator Cycle Sequencing Kit (Applied Biosystems) and primers synthesized on an Oligo 100 instrument (Beckman, Fullerton, CA). The *CgDN3* gene sequence (Fig. 2) was derived from two overlapping genomic clones. These two overlapping sequences were a PCR product amplified from genomic DNA with primers designed from the cDNA clone DN3-2 and a sequence obtained by PCR from a clone obtained by screening a genomic library with cDNA DN3-1. Primers designed from the termini of the DN3-2 cDNA insert, 5' - CCT CCT CTT TTT CCT CCT CGT C - 3' and 5' - GTC TTT CCT ATT TAC GGT TTC CCC - 3', were used in a PCR with genomic DNA as the template, and the PCR products cloned into pGEM-T. This genomic clone, named p3g4, was sequenced fully in both directions. A genomic library of *C. gloeosporioides* DNA (isolate UQ62) in λ EMBL-3 (He et al. 1996) was screened with the full-length cDNA clone DN3-2 to obtain a corresponding genomic clone. Genomic DNA corresponding to the 5' end of the DN3-2 cDNA and further upstream genomic sequences were amplified from the DNA prepared from one λ EMBL-3 clone with a primer designed from the DN3-2 cDNA sequence 5' - AAA GAG GAG GCT TCG GGA GTA C - 3' and a primer specific for the SP6 region in the λ EMBL-3 vector 5' - CGA TTT AGG TGA CAC TAT AG - 3'. The 1.8-kb product was subcloned with the Promega pGEM-T cloning kit and the resulting clone was named p3PROM and sequenced. This sequence was analyzed for known regulatory elements with the TESS data bases.

Southern and Northern hybridization analysis and RT-PCR.

DNA was extracted from *C. gloeosporioides* as described by Yoon et al. (1991). Southern blots were prepared with Hybond N⁺ (Amersham, Little Chalfont, UK) and 10 μg of genomic DNA (Sambrook et al. 1989). RNA for Northern analysis was extracted and Northern blots prepared as outlined in Masel et al. (1993). Hybridizations were carried out under high stringency conditions. Total RNA for RT-PCR was extracted from either cultured mycelia or infected leaves at 24-h intervals from 1 to 7 days after inoculation and also from a

control non-inoculated plant. RT-PCR was performed as described by Curtis et al. (1997). For RT-PCR of the *CgDN3* gene, the same primers used for amplification of the genomic fragment cloned in p3g4 described above were used.

Analysis of fungal transformants expressing promoter-GFP reporter fusions.

The *CgDN3* promoter region and part of the coding region were amplified from genomic DNA with the primer sequences 5' - AAT GAT GTG CGA GAT GCG G - 3' and 5' - TCA ATA GAG AGA GGA GAG GCG AC - 3'. The amplified DNA fragment was subcloned as plasmid pCDF1R1, and was sequenced to confirm its identity. This clone was used as a template in a PCR with the primers 5' - TCG ATA AGC TTG TGC GAG ATG CGG - 3' and 5' - AGA ATT GGC AGA TGA GAG CGG CC - 3'. This reaction amplified a 1,370-bp fragment with *Hind*III and *Bam*HI cloning sites at the termini that contained the *CgDN3* promoter region from positions 16 to 1376 shown in Figure 2. A vector named p35SmGFP was used as a source of a GFP reporter gene modified by removal of a cryptic intron that encoded a GFP with the addition of a amino terminal signal sequence and a carboxy-terminal endoplasmic reticulum retention signal (Haseloff et al. 1997). The *Cauliflower mosaic virus* (CaMV) 35S promoter of p35SmGFP was replaced with the *CgDN3* promoter fragment following excision with *Hind*III and *Bam*HI. This new construct, named pDN3:mgfp, represented a transcriptional fusion of the *CgDN3* promoter with the modified GFP coding region. As a positive control for the detection of strong constitutive expression of GFP, another vector was made whereby the *gpdA* promoter of *Aspergillus nidulans* was excised from the vector pNOM102 (Roberts et al. 1989) with *Eco*RI and *Nco*I. This fragment was cloned into the same sites in the vector pUbiGFP (Schenk et al. 1998) to replace the maize ubiquitin promoter. The resulting vector was named pgpdA:sgfp. Both pDN3:mgfp and pgpdA:sgfp were introduced into the fungus by the transformation method of Poplawski et al. (1997) by co-transformation with the fungal transformation vector pRCOS1 (Crowhurst et al. 1992) that carries an *hph* gene cassette. Hygromycin-resistant colonies were isolated and screened for GFP expression by microscopy. For transformants containing the pDN3:mgfp construct, mycelial fragments were transferred to a growth medium lacking nitrogen compounds to observe GFP fluorescence. Transformants were single spored and the presence of the transgenes in mycelia verified by PCR. A stable, GFP-expressing transformant obtained with each construct was then used for detailed microscopic analysis and infection studies. Transformants were observed directly on agar plates of rich media and rich media lacking nitrogen compounds as described above. For the analysis of promoter-GFP reporter during infection, leaves were detached and placed on cotton wool soaked in sterile water. The leaves were inoculated with conidial suspensions and incubated in the dark at 28°C in a sealed petri dish. Generally, infection sites were examined at 24 to 72 h after inoculation as whole fresh mounts. Petri plates and leaf material were examined for fluorescence with a Leica Z6 stereomicroscope fitted with the fluorescence GFP plus-filter module, and a mercury lamp light source (480/40 nm excitation filter, 505nm LP dichromatic beam splitting mirror, 510 LP barrier filter, and an S500/100 NP filter to remove chlorophyll fluo-

rescence). Slides mounts in water were examined with a Zeiss Axioskop fitted with a HBO 50 mercury lamp and fluorescein isothiocyanate (FITC) filter set (485/17 nm excitation filter, FT 500 beam splitting mirror and TBP 520 emission filter). Confocal microscopy was undertaken with a Bio-Rad MRC-500 confocal microscope with blue light excitation.

Disruption of *CgDN3* with the *hph* gene.

The fungal transformation vector pMSC-1 (Redman and Rodriguez 1994) was used as a source of an *hph* gene cassette with the *Escherichia coli* hygromycin resistance cistron regulated by promoter and terminator elements from the *TrpC* gene of *Aspergillus nidulans*. The *hph* gene was excised from pMSC-1 by digestion with *Bam*HI and *Sal*I, and subcloned into the corresponding sites of pBluescript II SK⁻ to increase the number of useful restriction sites. The resulting vector was named pBS*hph*. The *hph* gene was then excised with *Sal*I and *Spe*I and subcloned into the corresponding sites in p3g4 to form the vector p3*hph*. A 744-bp *Sal*I fragment excised from p3PROM was cloned into the *Sal*I site of p3*hph* to form the vector p3Ph*hph*. The orientation of the *C. gloeosporioides* fragments in p3Ph*hph* was checked by restriction analysis and confirmed by partial sequencing. This vector is shown diagrammatically in Figure 5. Transformation of *C. gloeosporioides* biotype B isolate UQ62 to hygromycin resistance was undertaken by the method of Poplawski et al. (1997). Hygromycin-resistant transformants were reisolated from single spores to ensure genetic uniformity, then used for Southern and Northern analysis and infection studies.

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