

# A *Magnaporthe grisea* Cyclophilin Acts as a Virulence Determinant during Plant Infection

Muriel C. Viaud, Pascale V. Balhadère, and Nicholas J. Talbot<sup>1</sup>

School of Biological Sciences, University of Exeter, Washington Singer Laboratories, Perry Road, Exeter EX4 4QG, United Kingdom

**Cyclophilins are peptidyl prolyl *cis-trans* isomerases that are highly conserved throughout eukaryotes and that are best known for being the cellular target of the immunosuppressive drug cyclosporin A (CsA). The activity of CsA is caused by the drug forming a complex with cyclophilin A and inhibiting the calmodulin-dependent phosphoprotein phosphatase calcineurin. We have investigated the role of *CYP1*, a cyclophilin-encoding gene in the phytopathogenic fungus *Magnaporthe grisea*, which is the causal agent of rice blast disease. *CYP1* putatively encodes a mitochondrial and cytosolic form of cyclophilin, and targeted gene replacement has shown that *CYP1* acts as a virulence determinant in rice blast. *Cyp1* mutants show reduced virulence and are impaired in associated functions, such as penetration peg formation and appressorium turgor generation. *CYP1* cyclophilin also is the cellular target for CsA in *Magnaporthe*, and CsA was found to inhibit appressorium development and hyphal growth in a *CYP1*-dependent manner. These data implicate cyclophilins as virulence factors in phytopathogenic fungi and also provide evidence that calcineurin signaling is required for infection structure formation by *Magnaporthe*.**

## INTRODUCTION

Rice blast is the most serious disease of cultivated rice and causes serious recurrent epidemics throughout the rice-growing regions of the world (Baker et al., 1997). The disease is caused by the ascomycete fungus *Magnaporthe grisea*, which produces specialized infection structures called appressoria to penetrate the leaves and stems of rice plants, allowing the fungus entry to the underlying tissue (for reviews, see Howard and Valent, 1996; Hamer and Talbot, 1998). Plant infection by appressoria is accomplished by the generation of enormous turgor pressure, which is translated into mechanical force to breach the plant cuticle (Howard et al., 1991). Once in the plant, the fungus develops bulbous secondary hyphae and quickly colonizes the host tissue (Bourett and Howard, 1990; Heath et al., 1990; Talbot et al., 1993). Damage to the rice crop results from either leaf blast, which kills or debilitates seedlings, or neck and panicle blast, which destroy the rice grain during the seed-setting stage (Ou, 1985). The economic cost of rice blast is significant, both in direct losses to the rice harvest and in the costs of control measures such as rice breeding programs, fungicide applications, and the development of new antifungal chemicals (Baker et al., 1997).

Developing novel mechanisms to control rice blast will require detailed understanding of both the disease and the developmental biology of *Magnaporthe*. Considerable progress has been made in identifying the genes required for elaboration of appressoria because the fungus is tractable to molecular genetics (Talbot, 1995; Howard and Valent, 1996), but far less is known about the later stages of plant infection and how the growth of the fungus within rice tissue is regulated. Appressorium formation on the rice leaf surface requires a cAMP signaling pathway that responds to inductive signals from the plant, including surface hydrophobicity and release of wax monomers from the cuticle (Lee and Dean, 1993; Mitchell and Dean, 1995; Gilbert et al., 1996; Xu et al., 1997). A mitogen-activated protein kinase (MAPK) pathway also is required for the differentiation of appressoria (Xu and Hamer, 1996), and the MAPK component of this signaling pathway is conserved in diverse fungal pathogens (Lev et al., 1999; Takano et al., 2000; Ruiz-Roldan et al., 2001). Appressorial turgor pressure then results from intracellular accumulation of very high concentrations of glycerol (de Jong et al., 1997), which provides the osmotic potential to draw water into the cell. Hydrostatic pressure develops because glycerol is retained in the appressorium as a result of the relatively impermeable cell wall, which is lined with a thick layer of melanin (Howard and Valent, 1996; de Jong et al., 1997). As a consequence, melanin-deficient mutants do not develop turgor and are nonpathogenic (Chumley and Valent, 1990). Appressorial turgor generation is accompanied by cAMP and

<sup>1</sup>To whom correspondence should be addressed. E-mail n.j.talbot@exeter.ac.uk; fax 44-1392-264668.

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*PMK1*-dependent mobilization of carbohydrate and lipid reserves, which accumulate in developing appressoria before being degraded rapidly in mature cells before plant infection (Thines et al., 2000; Weber et al., 2001).

To develop a better understanding of plant infection by Magnaporthe, we have initiated a number of studies to identify genes that play roles in appressorium function or in plant tissue invasion. These studies have involved mutagenesis and gene-tagging approaches (Balhadère et al., 1999; Balhadère and Talbot, 2001) or the selection of genes based on homology with known signaling components in yeast (Dixon et al., 1999). In this study, we used differential cDNA screening to identify an additional group of Magnaporthe genes that are highly expressed during plant infection (Talbot et al., 1993), and we report the characterization of one of these genes, *CYP1*, which encodes a cyclophilin.

Cyclophilins are a conserved family of proteins present in bacteria, fungi, plants, and animals and are best known for being the cellular target of the immunosuppressive drug cyclosporin A (CsA) (Marks, 1996). Cyclophilins also have peptidyl prolyl *cis-trans* isomerase activity and have been implicated in a wide variety of cellular processes, including the response to environmental stresses, cell cycle control, the regulation of calcium signaling, and the control of transcriptional repression (Sykes et al., 1993; Lu et al., 1996; Andreeva et al., 1999; Gotherl and Marahiel, 1999; Arévalo-Rodriguez et al., 2000).

We have investigated the role of *CYP1* using targeted gene replacement and have found that cyclophilin acts as a virulence determinant in rice blast disease. We show that  $\Delta cyp1$  cyclophilin mutants of Magnaporthe are impaired in plant infection and produce appressoria that do not develop full turgor. Additionally, we report that appressorium development shows very acute sensitivity to CsA in a cyclophilin-dependent manner, suggesting a role for the calcineurin regulation of appressorium development. Our findings parallel a very recent independent study that has established that cyclophilin is required for virulence of the human pathogenic fungus *Cryptococcus neoformans* (Wang et al., 2001). Together, these results implicate cyclophilins as virulence factors for both plant and animal diseases caused by fungi.

## RESULTS

### Identification of the *CYP1* Cyclophilin Gene from Magnaporthe

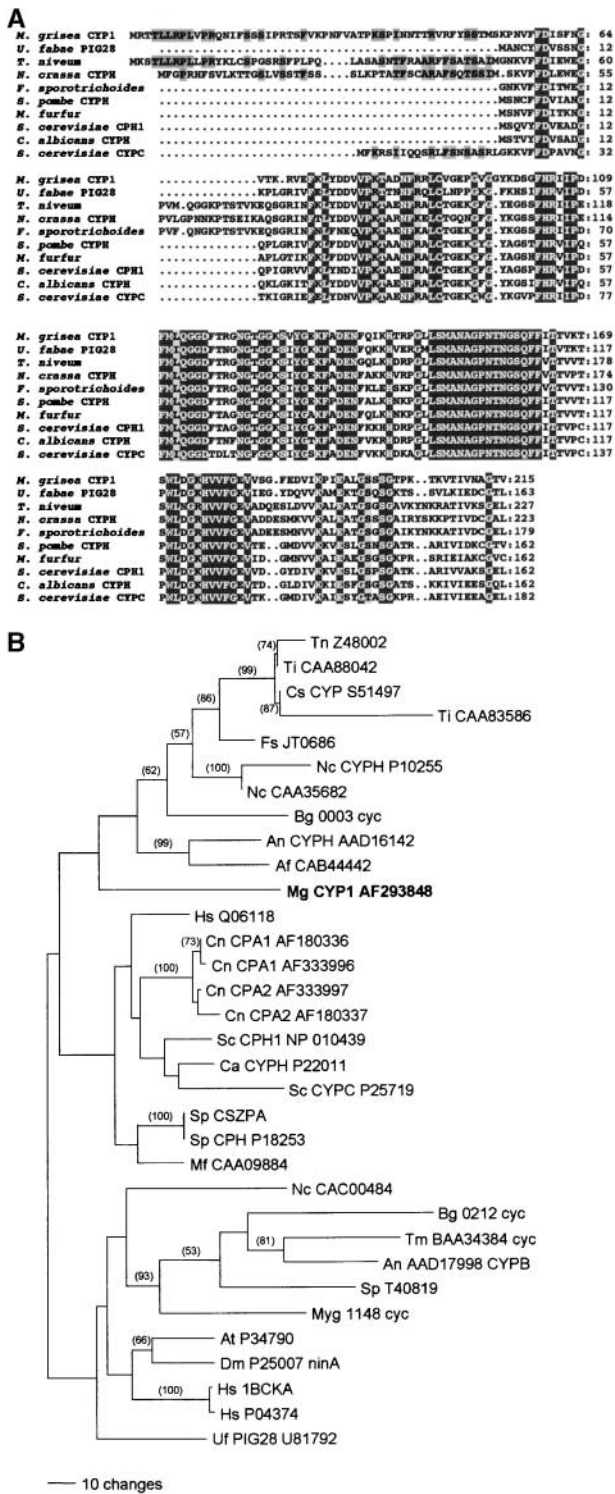
The *CYP1* cyclophilin gene was identified during a differential cDNA screening experiment that we performed to identify genes that are highly expressed during plant infection (Talbot et al., 1993). A cDNA clone corresponding to one of the most highly expressed genes was selected and sequenced. This revealed two putative translation initiation codons within the same open reading frame, predicting two

potential polypeptide products. The predicted proteins showed a high level of similarity to peptidyl prolyl *cis-trans* isomerases belonging to the cyclophilin family of immunophilins, as shown in Figure 1 (Marks, 1996; Andreeva et al., 1999). The longer open reading frame was 215 codons in length and putatively encodes a protein of 23.5 kD. The shorter form was 165 amino acids in length, being initiated at codon 51 of the longer open reading frame, and encodes a protein of 17.8 kD. The N-terminal extension of the 23.5-kD form of *CYP1* was similar to mitochondrial cyclophilin 20 from *Neurospora crassa* and *Tolypocladium niveum* (Figure 1A).

In *N. crassa*, it has been shown that a single gene, *CYPH*, encodes both mitochondrial cyclophilin 20 and cytoplasmic cyclophilin A and also has two initiation codons within a single open reading frame (Tropschug et al., 1988; Rassow et al., 1995). The organization of *CYP1*, therefore, is consistent with the gene encoding the mitochondrial and cytoplasmic forms of cyclophilin in Magnaporthe. Additionally, the N-terminal extension of *CYP1* contains eight positively charged amino acid residues and is rich in Ser and Thr residues. Therefore, it has characteristics of a mitochondrial target sequence (von Heijne, 1986), which was supported by analysis of the sequence with PSort II protein subcellular localization software (Nakai and Kanehisa, 1992), which predicted the probability of the protein being localized to the mitochondrion as 100%. The shorter 18-kD cyclophilin, meanwhile, lacks any signal peptide or organelle-targeting motifs and thus is likely to be the cytoplasmic cyclophilin A (52.2% probability based on PSort II). Phylogenetic analysis confirmed the classification of *CYP1* with cyclophilin A-type immunophilin genes from other filamentous fungi, as shown in Figure 1B.

To determine whether *CYP1* encodes both forms of cyclophilin, we performed a series of RNA and DNA gel blot experiments and reverse transcriptase-polymerase chain reaction (RT-PCR). In *N. crassa*, the *CYPH* gene is transcribed into two distinct mRNA species corresponding to each form of cyclophilin, indicating that the gene also has two distinct transcriptional initiation sites (Tropschug et al., 1988). To determine whether *CYP1* is expressed in the same manner, we first showed by DNA gel blot analysis that *CYP1* is present as a single copy in the Magnaporthe genome (data not shown). We then performed RNA gel blot analysis of Magnaporthe mycelium grown under different conditions, which revealed the presence of two distinct *CYP1* mRNA transcripts of ~990 and ~840 bp, as shown in Figure 2.

Next, we performed RT-PCR experiments using primer pairs specific to either the longer (mitochondrial) or shorter mRNAs of *CYP1*. We designed primers that not only were specific to each *CYP1* transcript but also spanned an intron, such that amplification from either genomic DNA or cDNA would give differently sized amplicons. This revealed the presence of both the longer and shorter transcripts in mRNA from Magnaporthe strain Guy11 (Figure 2B). Our results, therefore, suggest that *CYP1* is a single-copy gene that produces two transcripts encoding each cyclophilin (Tropschug



**Figure 1.** Predicted Amino Acid Sequence and Phylogenetic Analysis of the Magnaporthe CYP1 Gene.

**(A)** Alignment of the predicted CYP1 cyclophilin-encoding gene product with cyclophilins from *Uromyces fabae*, *Tolypocladium*

et al., 1988). CYP1 was expressed at a high level in Magnaporthe mycelium grown in rice and also after exposure to starvation or hyperosmotic stress (Figure 2). CYP1 expression was unaltered in a  $\Delta pmk1$  MAPK mutant, although variation in the abundance of the longer (mitochondrial) mRNA species was observed under conditions of starvation stress. CYP1, therefore, may be under the negative control of the PMK1 MAPK pathway, at least during starvation stress (Xu and Hamer, 1996).

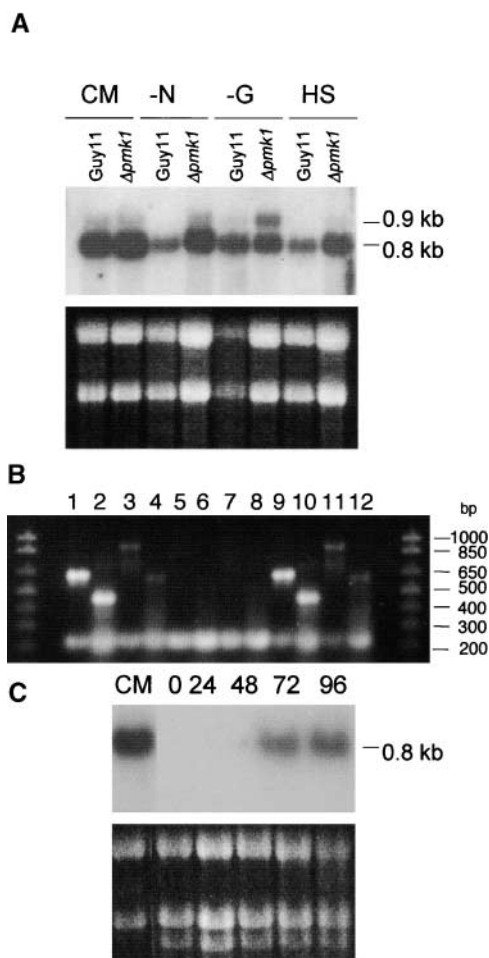
To examine the role of CYP1 in pathogenic development, we investigated CYP1 expression during rice infection. Rice plants were infected with Magnaporthe, and the disease was allowed to proceed for 24, 48, 72, and 96 hr. At each time point, total RNA was extracted, and gel blot analysis showed high levels of CYP1 expression during plant infection, particularly during later stages. We were able to observe the presence of the shorter transcript only during these experiments and in RT-PCR (data not shown). We conclude that CYP1 encodes two forms of cyclophilin in Magnaporthe and is highly expressed during both vegetative growth and pathogenic development.

**CYP1 Encodes a Virulence Determinant for Rice Blast Disease**

On the basis of the expression pattern of CYP1 and its lack of obvious regulation by the PMK1 MAPK, we reasoned that

*niveum*, *Neurospora crassa*, *Fusarium sporotrichoides*, *Schizosaccharomyces pombe*, *Saccharomyces cerevisiae* (CPH1 and CYPC), and *Candida albicans* (CYPH). The sequences were aligned with CLUSTALW (Thompson et al., 1994). Identical amino acids are highlighted against a black background, conserved residues are shown on a dark gray background, and similar amino acids are shown on a light gray background. CYP1, *T. niveum* CYP, and the *N. crassa* CYPH genes contain N-terminal extensions encoding a mitochondrial form of the cyclophilin.

**(B)** For phylogenetic analysis, predicted amino acid sequences from 33 different cyclophilin genes were compared using a heuristic search with the branch-swapping and nearest-neighbor interchange options in the program PAUP\* version 4.0s (Swofford, 2000). Branch strengths were tested by 100 repetitions of the bootstrap algorithm with branch swapping (numbers in parenthesis show the percentage bootstrap value). Lengths of branches are shown. The tree was rooted using the midpoint method. Abbreviations and numbers correspond to gene names and GenBank accession numbers, respectively. Organisms are as follows: Tn, *T. niveum*; Ti, *Tolypocladium inflatum*; Cs, *Cordyceps subsessilis*; Fs, *F. sporotrichoides*; Nc, *N. crassa*; Bg, *Botrytis cinerea*; Tm, *Trichophyton mentagrophytes*; An, *Aspergillus nidulans*; Af, *Aspergillus fumigatus*; Mg, *M. grisea*; Hs, *Homo sapiens*; Cn, *Cryptococcus neoformans*; Sc, *S. cerevisiae*; Ca, *Candida albicans*; Sp, *S. pombe*; Mf, *Malassezia furfur*; Myg, *Mycosphaerella graminicola*; At, *Arabidopsis thaliana*; Dm, *Drosophila melanogaster*; Uf, *U. fabae*.



**Figure 2.** *CYP1* Encodes Two mRNA Transcripts and Is Highly Expressed in Vegetative Growth in Culture and during Rice Blast Disease.

**(A)** Mycelial cultures of Magnaporthe Guy11 and the  $\Delta pmk1$  mutant nn95 strain (Xu and Hamer, 1996) were grown in rich growth medium (CM) for 48 hr, and mycelium was removed and transferred to growth medium lacking nitrate (-N) or glucose (-G) or supplemented with 0.4 M NaCl (hyperosmotic stress [HS]) or transferred to CM in the control experiment. Cultures were grown for another 24 hr, RNA was removed, and RNA gel blots were prepared. Blots were probed with a 1.5-kb EcoRI-XhoI insert from the *CYP1* cDNA clone pMV1. Transcripts of  $\sim 990$  and  $\sim 840$  bp were detected.

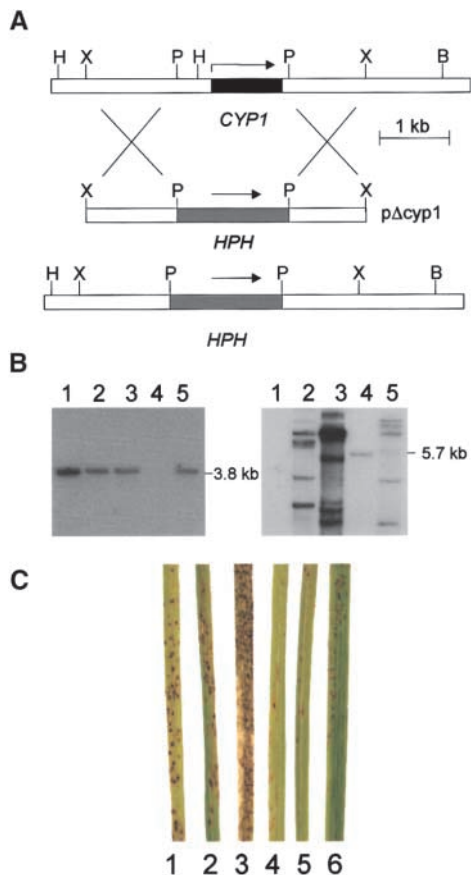
**(B)** RT-PCR amplification of *CYP1* using gene-specific primer pairs. Total RNA was extracted from Magnaporthe and reverse transcribed to cDNA using an oligo(dT) primer. PCR was performed on the resulting cDNA templates (lanes 2, 4, 6, 8, 10, and 12) and genomic DNA templates (lanes 1, 3, 5, 7, 9, and 11). Primer pairs were designed to amplify intron-containing fragments from genomic DNA and shorter intron-free amplicons from cDNA templates. Primer pair CC5-1 and CC3-1 specific to the shorter *CYP1* transcript were predicted to amplify a 599-bp fragment from genomic DNA and a 400-bp fragment from cDNA, respectively. Primer pair MC5-1 and CC3-1 were predicted to amplify an 874-bp fragment from DNA and a 580-bp fragment from cDNA, respectively. Lanes 1 to 4, amplification from

*CYP1* might be important in the stages of pathogenic development after appressorium formation. PMK1 is the central regulator of appressorium formation (Xu and Hamer, 1996), and we were particularly interested in identifying gene functions that affect plant infection and plant tissue invasion by Magnaporthe. To determine the significance of *CYP1* in disease development, we performed a one-step gene replacement, as shown in Figure 3. The resulting  $\Delta cyp1$  null mutants were selected for further study based on the absence of hybridization to a *CYP1* gene probe and the presence of a single copy of the *HPH* selectable marker gene cassette (Figure 3B). We also confirmed the absence of both *CYP1* mRNAs in a  $\Delta cyp1$  null mutant, as shown in Figure 2B. A  $\Delta cyp1$  null mutant transformant, TR33, was selected and used to inoculate the blast-susceptible rice cv CO-39. Representative results of these plant infections are shown in Figure 3C.

The mean disease lesion density was  $17.47 \pm 9.54$  lesions per 5 cm of leaf for the wild-type Magnaporthe strain Guy11 and  $5.67 \pm 2.83$  for the  $\Delta cyp1$  mutant TR33. The difference in virulence was statistically significant when analyzed using Student's *t* test ( $t = 3.55$ ,  $df = 18$ ,  $P < 0.05$ ). The reduction in virulence led us to investigate the growth and development of the  $\Delta cyp1$  mutant TR33 in detail. First, we observed vegetative mycelial growth on rich growth medium (Talbot et al., 1993) to determine whether the pathogenicity defect of  $\Delta cyp1$  mutants was caused by reduced fitness. Vegetative growth was uniform, however, in the  $\Delta cyp1$  mutant TR33, which showed a mean growth of  $7.96 \pm 0.35$  cm after 14 days, compared with  $7.81 \pm 0.28$  cm for Guy11 ( $\chi^2 = 0.08$ ,  $df = 11$ ,  $P > 0.05$ ). Conidial germination rates and the ability to elaborate appressoria also were unaltered in the  $\Delta cyp1$  mutant (data not shown), although conidiogenesis in the  $\Delta cyp1$  mutant TR33 was reduced significantly (approximately threefold) compared with that of Guy11 ( $\chi^2 = 83.92$ ,  $df = 5$ ,  $P < 0.001$ ). The  $\Delta cyp1$  mutant TR33 did not show any enhanced sensitivity to heat shock, starvation, or hyperosmotic stress compared with the wild type (data not shown).

Magnaporthe wild-type strain Guy11; lanes 5 to 8, amplification from  $\Delta cyp1$  strain TR33; lanes 9 to 12, amplification from *CYP1*-complemented  $\Delta cyp1$  transformant MV42. The 200-bp amplicon is a fragment of the Magnaporthe actin gene that was amplified as a positive control from every template.

**(C)** Twenty-day-old rice seedlings of cv CO-39 were inoculated with conidia of Magnaporthe strain Guy11, and rice blast disease was allowed to progress until symptoms became apparent 96 hr later. RNA was extracted from rice seedlings at 0, 24, 48, 72, and 96 hr. RNA gel blots were probed with a 1.5-kb EcoRI-XhoI insert from the *CYP1* cDNA clone pMV1. An equivalent RNA gel blot of fractionated RNA from a Magnaporthe mycelial culture (CM) was hybridized to the same probe under the same conditions, and the autoradiograph was exposed for an equal period.



**Figure 3.** Targeted Replacement of the *CYP1* Gene Results in a Reduction in Virulence.

**(A)** Restriction map of the *CYP1* locus showing the orientation of the *CYP1* open reading frame. A 4.3-kb *XhoI* fragment spanning the locus was subcloned to create *pCYP1X*, and a 1.1-kb *PstI* fragment, which contained the entire *CYP1* open reading frame, was removed. This was replaced with a 1.0-kb *PstI* fragment containing a hygromycin B resistance gene cassette (*HPH*). The construct *pΔcyp1* was linearized and transformed into *Magnaporthe* Guy11. Transformants were selected that had undergone homologous recombination. B, BamHI; H, HindIII; P, *PstI*; X, *XhoI*.

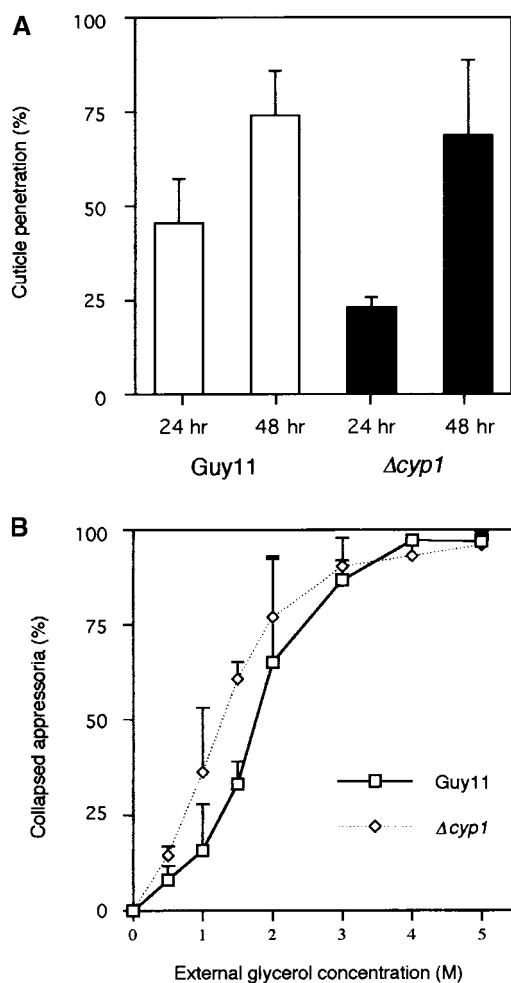
**(B)** DNA gel blot analysis of *pΔcyp1* *Magnaporthe* transformants. Genomic DNA was digested with BamHI and HindIII and probed with the 1.1-kb *PstI* *CYP1* fragment (left) and the 1-kb *HPH* cassette (right). Lane 1, Guy11; lanes 2, 3, and 5, transformants showing ectopic insertion of *pΔcyp1*; lane 4, *Δcyp1* transformant TR33.

**(C)** Rice blast symptoms produced by *Δcyp1* mutant TR33 and the isogenic wild-type strain Guy11 on rice cv CO-39. Leaves 1 and 2 were from plants that had been inoculated with a suspension of Guy11 conidia at a concentration of  $10^4$  conidia/mL. Leaf 3 was from a plant inoculated with a suspension of Guy11 conidia at a concentration of  $10^5$  conidia/mL. Leaves 4 and 5 were from plants that had been inoculated with a suspension of *Δcyp1* mutant TR33 conidia at a concentration of  $10^4$  conidia/mL. Leaf 6 was from a plant inoculated with a suspension of *Δcyp1* TR33 conidia at  $10^5$  conidia/mL.

Because appressoria formed normally in *Δcyp1* mutants, we decided to investigate whether the infection structures produced were fully functional. Appressoria were allowed to form on epidermal layers from rice leaves, and the frequency of penetration peg formation was determined, as shown in Figure 4. After 24 hr of incubation, only  $23 \pm 2.7\%$  of *Δcyp1* mutant appressoria had produced penetration pegs, compared with  $45.4 \pm 11.8\%$  of Guy11 appressoria (Figure 4A). After 48 hr, however, the penetration frequency of *Δcyp1* appressoria was almost as high as that of Guy11 appressoria, indicating that penetration peg formation was delayed in the mutant rather than arrested completely. Delayed penetration peg formation in *Δcyp1* mutant infections was accompanied by a distinctive defense response from the rice epidermal cells, which produced brown coloration at the site of penetration (data not shown).

Appressorium-mediated rice infection by *Magnaporthe* is a turgor-driven process; therefore, we used an incipient cytorrhysis assay to measure appressorial turgor in the *Δcyp1* mutant (Howard et al., 1991; de Jong et al., 1997). The assay was performed by first incubating uniform suspensions of conidia in 50- $\mu$ L water drops on plastic cover slips to undergo appressorium formation. After maturation of appressoria (24 hr), the surrounding water droplet was removed carefully and replaced with a solution of glycerol ranging in concentration from 0.5 to 5.0 M. The proportion of appressoria collapsing at each concentration of glycerol then was calculated and used to estimate the turgor of appressoria, as shown in Figure 4B (Dixon et al., 1999). We found that 1.5 M glycerol was sufficient to collapse  $30 \pm 3\%$  of appressoria of Guy11 and  $62 \pm 4\%$  of *Δcyp1* appressoria ( $\chi^2 = 38.2$ ,  $df = 2$ ,  $P < 0.05$ ). This result indicates that appressorial turgor in *Δcyp1* mutants is reduced significantly compared with that in an isogenic *Magnaporthe* strain.

Because of the observed reduction in appressorial turgor in the *Δcyp1* mutant, we decided to perform cytological analysis of appressoria in more detail. Recently, it was reported that glycogen and lipid reserves are localized in developing *Magnaporthe* appressoria under the control of the cAMP response pathway, leading to turgor generation and plant infection (Thines et al., 2000; Weber et al., 2001). We found that the distribution of glycogen reserves in *Δcyp1* mutant conidia and appressoria, as observed by light microscopy (Thines et al., 2000), was identical to that in the wild-type *Magnaporthe* strain Guy11 (data not shown), although significantly less lipid was present. Conidia from Guy11 contained large numbers of lipid bodies that stained brightly with Nile red, as shown in Figure 5B. In contrast, Nile red uptake by conidia of the *Δcyp1* mutant TR33 suggested the presence of less lipid (Figure 5D), which was found predominantly in the cortical cytoplasm at the periphery of a large central vacuole. During appressorium development, lipid reserves localized to appressoria in Guy11 (Figure 5F), and although the same distribution of lipid bodies was observed in TR33, there was significantly less lipid present when assessed by Nile red staining (Figure 5H).



**Figure 4.** The  $\Delta cyp1$  Mutant Is Impaired in Cuticle Penetration and Appressorium Turgor Generation.

**(A)** Proportions of appressoria that were able to penetrate rice cuticles of cv CO-39 after 24 and 48 hr of maturation. Cuticle penetration was observed by acid fuchsin staining of cleared cuticle and epidermal strips, as described by Balhadère et al. (1999). Each bar represents the mean value of three independent experiments, and error bars show standard deviations.

**(B)** Appressorium turgor was measured by incipient cytorrhysis. Appressoria were allowed to form on plastic cover slips for 24 hr, and the proportion of appressoria that collapsed after exposure to solutions between 0 and 5.0 M glycerol was recorded. Each data point is the mean of three independent observations, and the error bars represent standard deviations.

Light microscopy also confirmed that  $\Delta cyp1$  mutant appressoria were fully melanin pigmented in the same way as Guy11 (Figures 5E and 5G), indicating that the reduction in turgor was not attributable to incorrect cell wall formation. Transmission electron microscopy revealed the presence of

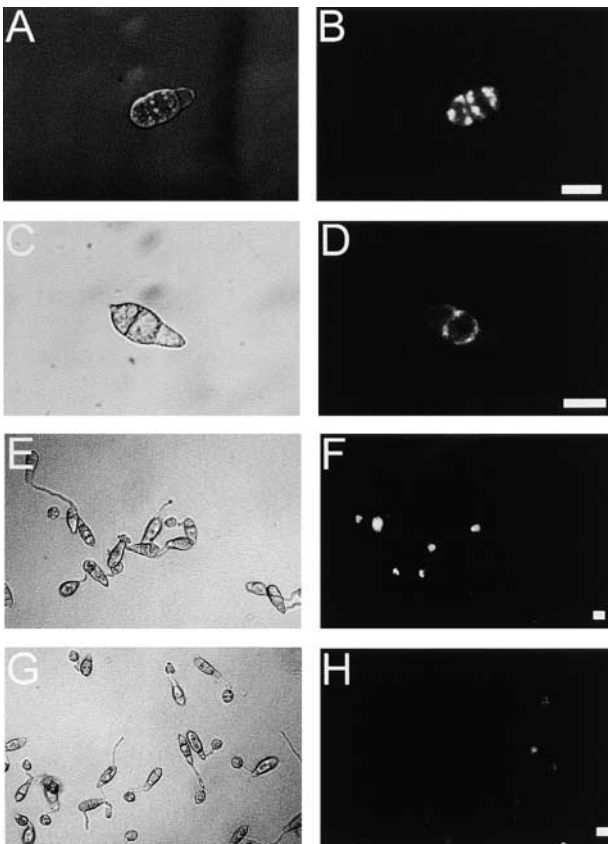
numerous small lipid bodies in Guy11, as shown in Figure 6, and although  $\Delta cyp1$  mutant conidia also contained lipid bodies, they were less abundant and the conidia appeared to be more highly vacuolated (Figure 6). We conclude that the  $\Delta cyp1$  mutation does not affect overall fitness in Magnaporthe, as measured by growth rates and spore germination frequency, but it does affect the ability of the fungus to cause rice blast disease. The cyclophilin mutation also causes a significant delay in rice cuticle penetration and a reduction in cellular turgor.

### **CYP1-Encoded Cyclophilin Is the Cellular Target for CsA in Magnaporthe**

Cytoplasmic cyclophilin A is well known as the target for the immunosuppressive drug CsA in mammalian cells (for review, see Marks, 1996). Immunosuppression by CsA occurs because of the inhibition of T cell activation. In humans, T cell activation is induced after antigen presentation to the T cell receptor and involves nuclear localization of a transcription factor, the nuclear factor for activated T cells (NFAT), under the control of the calcium/calmodulin-regulated protein phosphatase calcineurin. CsA binds to cyclophilin A in the cytoplasm, and the resulting cyclophilin A–CsA complex targets calcineurin, thereby preventing its action (Flanagan et al., 1991; Liu et al., 1991; Kunz and Hall, 1993; Marks, 1996). In addition to causing immunosuppression, however, CsA also is known to be a potent antifungal agent and has fungicidal activity against a wide range of species. Studies in *N. crassa* and the human pathogen *C. neoformans* suggest that calcineurin also is the target of CsA in fungi, and impairment of calcineurin function has been shown to cause defects in the apical growth of hyphae along with gross morphological changes in *N. crassa* (Odom et al., 1997; Prokisch et al., 1997; Wang et al., 2001). Moreover, in *A. nidulans*, it has been shown genetically that calcineurin is essential for viability (Rasmussen et al., 1994).

Therefore, we decided to test whether *CYP1* encodes the cellular target for CsA in Magnaporthe and to determine the effect of the drug on hyphal development. In plate assays, we found that CsA exhibited potent antifungal activity against Magnaporthe and severely inhibited the vegetative growth of Guy11 at concentrations  $>100 \mu\text{g/mL}$ , as shown in Figure 7A. In contrast, the  $\Delta cyp1$  TR33 transformant grew normally, even in the presence of  $3000 \mu\text{g/mL}$  CsA. This finding indicates that the fungicidal activity of CsA requires the *CYP1*-encoded cyclophilin.

The potent fungicidal activity of CsA is known to be the result of calcineurin targeting in the human pathogenic fungus *C. neoformans*, in which calcineurin has been shown to be required for virulence (Odom et al., 1997; Fox et al., 2001). Calcium- and calmodulin-dependent signaling also have been implicated in appressorium formation in Magnaporthe, based on pharmacological studies; calcium chelators and calmodulin antagonists, for instance, inhibit appressorium formation



**Figure 5.** Cellular Distribution of Lipid Droplets during Appressorium Morphogenesis by Magnaporthe Strain Guy11 and the  $\Delta cyp1$  Mutant TR33.

Conidia were allowed to germinate on glass slides in water drops and form appressoria. Sample preparations were removed at intervals during a 12-hr period and stained for the presence of triacylglycerol using Nile red, as described previously (Thines et al., 2000). Photographs were taken 0 and 12 hr after inoculation. Hoffman modulation contrast images (left) and epifluorescence images of Nile red-stained material (right) are presented.

(A) and (B) Guy11 conidium at 0 hr.

(C) and (D)  $\Delta cyp1$  mutant TR33 conidium at 0 hr.

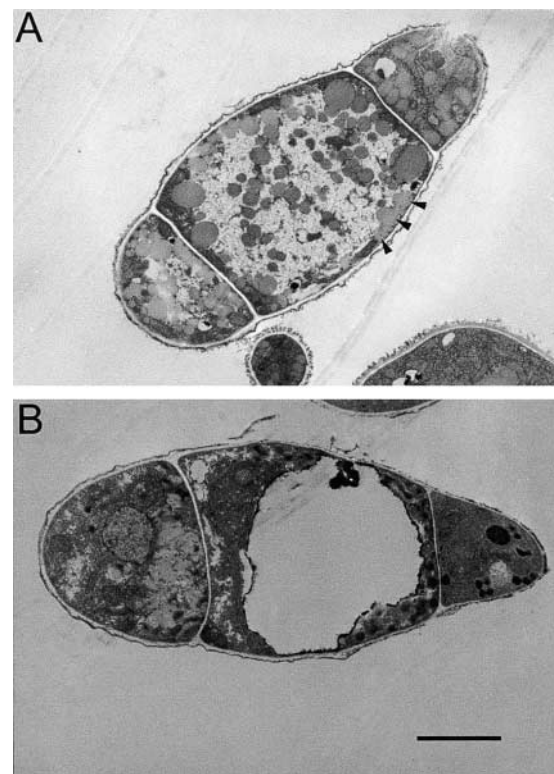
(E) and (F) Guy11 developing appressoria after 12 hr.

(G) and (H)  $\Delta cyp1$  mutant TR33 developing appressoria after 12 hr. Bars = 10  $\mu\text{m}$  for all panels.

(Lee and Lee, 1998; Liu and Kolattukudy, 1999), whereas diacylglycerol has been shown to remediate appressorium-deficient (*pth11*) mutants (DeZwaan et al., 1999).

Because of the effects of CsA on Magnaporthe hyphal development, we decided to determine the effect of the drug on the elaboration of appressoria. Conidia from Guy11 and the  $\Delta cyp1$  mutant TR33 were incubated in water on hydrophobic plastic cover slips in the presence of between 1 and 20  $\mu\text{g}/\text{mL}$  CsA. Appressorium formation then was moni-

tored over a 24-hr period. The frequency of conidial germination was almost identical in Guy11 ( $97.33 \pm 1.03\%$  in 24 hr) and the  $\Delta cyp1$  mutant TR33 ( $96.17 \pm 3.54\%$ ), even after exposure to 20  $\mu\text{g}/\text{mL}$  CsA, and the rate of germ tube extension also was unaffected (data not shown). The ability of conidia to elaborate appressoria, however, was severely affected in the wild-type Magnaporthe strain Guy11, even at concentrations as low as 1  $\mu\text{g}/\text{mL}$ , which do not affect hyphal development (Figures 7B and 7C). CsA, therefore, inhibits appressorium formation at concentrations that are not fungicidal to Magnaporthe, and the CsA-dependent inhibition of appressorium development requires the presence of the *CYP1* cyclophilin.



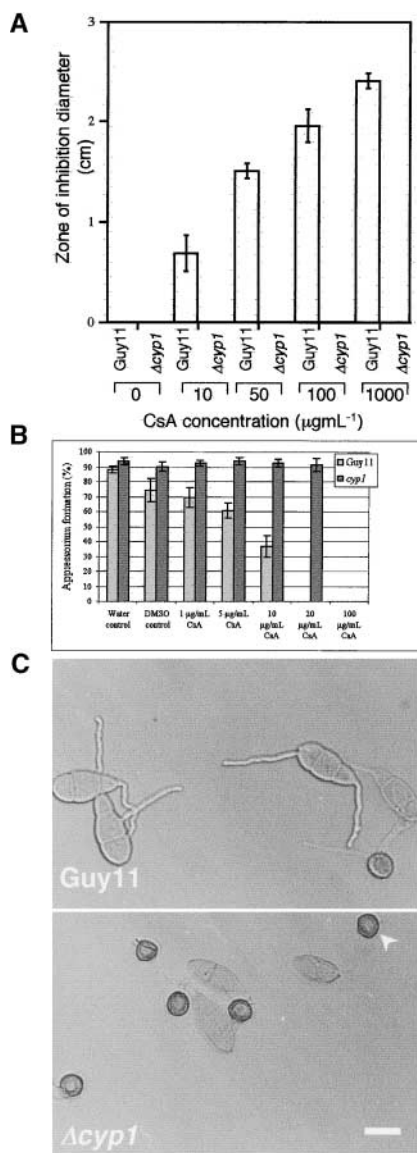
**Figure 6.** Ultrastructure of Conidia Produced by Magnaporthe Strain Guy11 and the  $\Delta cyp1$  Mutant TR33.

Conidia were harvested from 12-day-old plate cultures of Magnaporthe and prepared for transmission electron microscopy using chemical fixation and ultrathin sectioning, as described by Weber et al. (2001).

(A) Median section of a three-celled conidium of Guy11 showing the distribution of numerous lipid bodies (arrowheads). A vacuole with heterogeneous contents fills much of the central cell.

(B) Median section of a three-celled conidium of the  $\Delta cyp1$  mutant TR33 showing a large central vacuole and fewer lipid bodies, a small number of which are visible around the periphery of the central cell.

Bar in (B) = 5  $\mu\text{m}$  for (A) and (B).



**Figure 7.** CYP1 Mediates Sensitivity to CsA in Magnaporthe.

**(A)** Vegetative growth inhibition of Magnaporthe by CsA was assayed by inoculating  $3 \times 10^3$  conidia onto the surface of a CM agar plate. Filter paper discs containing CsA in 1% DMSO were placed on the culture, and the zones of growth inhibition were observed. The bar chart shows the diameter of the zone of inhibition after exposure to four concentrations of CsA. Each bar represents the mean of three independent replications of the experiment with three plates per experiment, and the error bars represent standard deviations.

**(B)** Effect of CsA on appressorium development by Magnaporthe. Each bar represents the mean of three independent experiments, and the error bars represent standard deviations.

**(C)** Magnaporthe appressorium formation on plastic cover slips after exposure to 10  $\mu\text{g/mL}$  CsA by Guy11 and  $\Delta\text{cyp1}$  mutant TR33. A dome-shaped appressorium is marked with an arrowhead. Bar = 10  $\mu\text{m}$ .

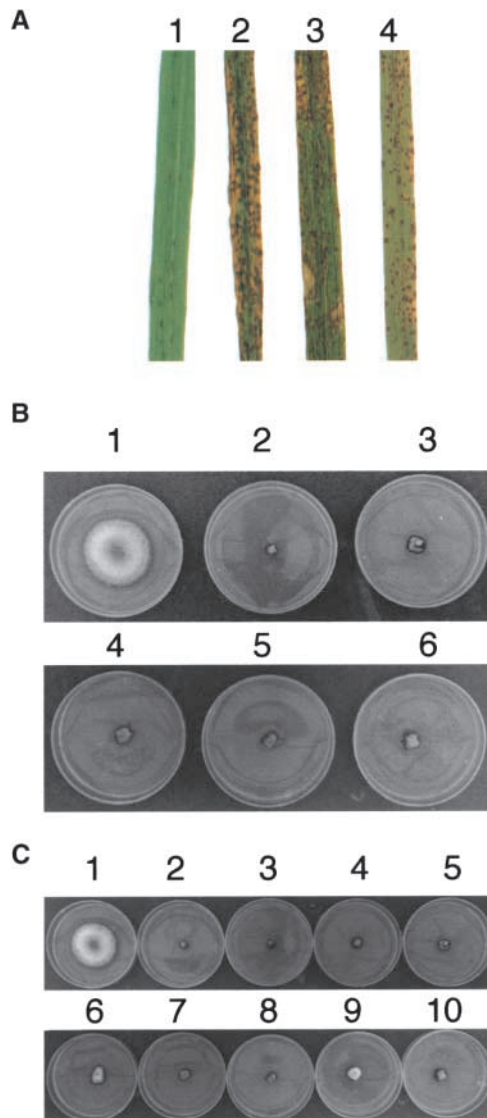
### Reintroduction of CYP1 Restores Virulence and CsA Sensitivity

As a result of the number of distinct phenotypes associated with the  $\Delta\text{cyp1}$  mutation, we performed a comprehensive complementation analysis to ensure that all of the phenotypes we observed were attributable to the cyclophilin gene replacement. Complementation tests were performed by introducing either a 1.1-kb PstI fragment spanning the CYP1 gene or a larger 4.3-kb XhoI genomic fragment (Figure 3) into the  $\Delta\text{cyp1}$  mutant TR33. Transformants were selected, and the presence of a single copy of the CYP1 genes was confirmed by DNA gel blot analysis (data not shown). We also confirmed the expression of both CYP1 transcripts in RT-PCR experiments, as shown in Figure 2B. Rice blast disease assays were performed by spraying uniform concentrations of conidia on rice seedlings of cv CO-39 and assessing disease symptoms after 5 days. Representative leaves from seedlings are shown in Figure 7A.

Seedlings sprayed with conidia of Magnaporthe  $\Delta\text{cyp1}$  mutant strain TR33 had relatively few disease lesions (Figure 8A, leaf 1) compared with those sprayed with Guy11 conidia (Figure 8A, leaf 2). Rice plants inoculated with conidia from transformants carrying either the 1.1-kb PstI CYP1 fragment (leaf 3) or the 4.3-kb XhoI CYP1 fragment (leaf 4) exhibited normal rice blast disease symptoms. Sensitivity to CsA also was restored by the introduction of either CYP1 genomic fragment, as shown in Figures 8B and 8C. All other  $\Delta\text{cyp1}$  mutant phenotypes reported were assessed in transformants and shown to be complemented by the presence of CYP1 (data not shown). The CYP1 cyclophilin, therefore, is a virulence determinant in Magnaporthe involved in appressorium-mediated infection and is required to mediate the inhibitory effects of CsA on fungal development.

### DISCUSSION

Cyclophilins are peptidyl prolyl *cis-trans* isomerases of the immunophilin family, which also includes the FK506 binding proteins and parvulins, and are highly expressed in many eukaryotic cell types (Kunz and Hall, 1993; Marks, 1996). The enzymatic action of cyclophilins is to direct and accelerate protein folding by catalyzing the isomerization of peptide bonds that precede Pro residues (Fischer and Schmid, 1990). Peptide bonds exist in either *cis* or *trans* isomeric conformations, and for all amino acids except Pro, the peptide bond is produced in its *trans* form because of instability in the *cis* configuration. In the case of peptide bonds preceding Pro residues, however, the peptide bond is equally stable in either the *cis* or the *trans* conformation, and often it is in the *cis* configuration. Peptidyl prolyl bonds are thought to be synthesized by ribosomes in the *trans* form and then either isomerize spontaneously or are altered catalytically to the *cis* form by peptidyl prolyl *cis-trans* isomerases (EC



**Figure 8.** Reintroduction of *CYP1* into a  $\Delta cyp1$  Mutant Restores Virulence and CsA Sensitivity.

**(A)** Rice blast assays were performed by spraying conidial suspensions of uniform concentration ( $10^5$  conidia/mL) onto seedlings of rice cv CO-39. Leaf 1 is from a plant inoculated with  $\Delta cyp1$  mutant TR33. Leaf 2 is from a plant inoculated with the isogenic wild-type strain Guy11. Leaf 3 is from a plant inoculated with MV42, a transformant of  $\Delta cyp1$  mutant TR33 that carries a single copy of a 1.1-kb PstI fragment of *CYP1*. Leaf 4 is from a plant inoculated with MV72, a transformant of  $\Delta cyp1$  mutant TR33 that carries a single copy of a 4.3-kb XhoI fragment of *CYP1*. CsA sensitivity assays were performed by incubating mycelial plugs of Magnaporthe on CM agar medium containing 100  $\mu\text{g}/\text{mL}$  CsA and incubating plates at 24°C for 5 days.

**(B)** Plate 1 was inoculated with  $\Delta cyp1$  mutant TR33, plate 2 was inoculated with Guy11, and plates 3 to 6 were inoculated with transformants MV37, MV39, MV40, and MV42, respectively, each of which carries a single copy of a 1.1-kb PstI fragment of *CYP1*.

5.2.1.8) during protein folding (Fischer and Schmid, 1990). Prolyl isomerization has been proposed to be a rate-limiting step in protein folding, suggesting that cyclophilins may play key regulatory roles in the functions of diverse proteins. Multiple cyclophilins exist in eukaryotic organisms and are located throughout the cell, for example, in the cytoplasm, mitochondria, and endoplasmic reticulum. Because of their widespread conservation among very diverse organisms and their distribution throughout different cellular domains, cyclophilins are believed to perform a variety of fundamental cell functions. In yeast, however, which contains eight cyclophilin genes, mutants lacking one or more cyclophilins have few obvious phenotypes (Dolinski et al., 1997).

In this study, we have shown that a cyclophilin is an important virulence determinant in a plant pathogenic fungus. Absence of the *CYP1*-encoded cyclophilin in Magnaporthe resulted in a lower rate of plant infection, based on the number of observed disease lesions, and affected the function of appressoria, which did not penetrate the leaf cuticle efficiently and were unable to generate full turgor. Deletion of *CYP1* did not affect the vegetative growth of Magnaporthe in culture, but the development of conidia was affected. The *CYP1* cyclophilin, therefore, appears to play a number of distinct cellular roles in Magnaporthe, influencing the development of asexual reproductive structures and cellular turgor generation in appressoria. The reduced virulence of  $\Delta cyp1$  mutants probably is attributable at least in part to the reduction in appressorium turgor and the consequent delay in cuticle penetration; even small reductions in appressorial turgor have been shown to prevent cuticle penetration by Magnaporthe (Howard et al., 1991). Furthermore, any delay in cuticle penetration allows the opportunity for the successful deployment of plant defenses (Xu et al., 1998). However, we cannot exclude the possibility that other virulence-related functions also are affected by the absence of *CYP1* cyclophilin, such as penetration peg development and invasive hyphal growth.

Appressorium turgor generation in Magnaporthe involves the rapid accumulation of glycerol, which is retained in the cell by the presence of a relatively impermeable melanin wall layer (de Jong et al., 1997). Because  $\Delta cyp1$  mutants produced fully melanized appressoria, it is unlikely that the cyclophilin regulates melanin biosynthesis or solute retention. The observation that conidia and infection structures of  $\Delta cyp1$  mutants contain less lipid, however, indicates that

**(C)** Plate 1 was inoculated with  $\Delta cyp1$  mutant TR33, plate 2 was inoculated with Guy11, and plates 3 to 10 were inoculated with transformants MV44, MV67, MV70, MV72, MV73, and MV74, respectively, each of which carries a single copy of a 4.3-kb XhoI fragment of *CYP1*.

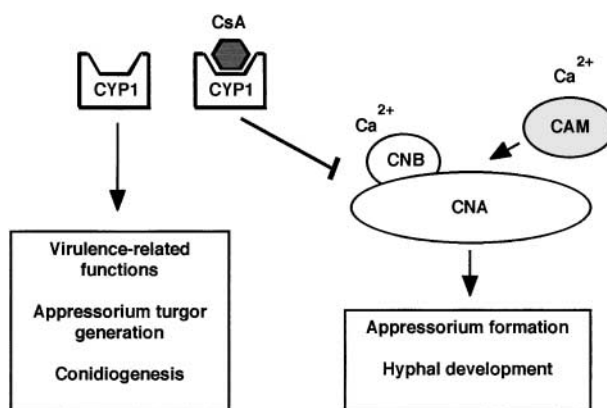
the synthesis of glycerol within the appressoria may be delayed, explaining the reduction in cellular turgor. In this regard, although it is tempting to speculate that *CYP1* may serve a very specific function in appressorium formation by regulating lipid biosynthesis and the mobilization required for glycerol generation in appressoria, it is equally likely that the reduction in appressorium turgor in  $\Delta cyp1$  mutants results from a more general loss of the control of gene expression required during appressorium maturation. In yeast, for example, cyclophilin A can influence transcription repression and gene silencing by acting on the histone deacetylase complex (Arévalo-Rodríguez et al., 2000).

Recently, two cyclophilin A–encoding genes were identified from the human pathogenic fungus *C. neoformans* (Wang et al., 2001) and shown to play partially overlapping and divergent roles in cell growth, mating, and virulence. The *Cpa1* and *Cpa2* cyclophilin A proteins share functions in cell growth and virulence, and *cpa1 cpa2* double mutants are severely attenuated for virulence and inviable at high temperatures. They also have divergent cellular roles, however; single *cpa1* mutants are inviable at high temperatures and reduced in virulence, whereas *cpa2* mutants are not temperature sensitive and are fully virulent. Interestingly, both *Cpa1* and *Cpa2* mediate sensitivity to CsA, indicating that either protein can form a CsA–cyclophilin A complex and inactivate calcineurin (Wang et al., 2001).

In this study, we have shown that *CYP1* encodes the cellular target for CsA in Magnaporthe, because deletion of the cyclophilin was sufficient to bestow high-level resistance to CsA. In addition to the effects of CsA-mediated calcineurin inhibition on hyphal development, which has been observed in other filamentous fungi (Rasmussen et al., 1994; Prokisch et al., 1997), we found that appressorium formation was acutely sensitive to exogenous CsA, being impaired at concentrations that were not fungicidal and that did not affect germ tube elongation. This observation provides evidence that calcineurin is required for appressorium morphogenesis. Calmodulin-dependent signaling has been implicated in the control of appressorium formation, based on inhibitor studies using calcium chelators and calcium channel modulators and specific expression of the calmodulin gene during appressorium formation (Lee and Lee, 1998; Liu and Kolattukudy, 1999). Inhibition of phospholipase C activity using neomycin, for example, causes pronounced inhibition of appressorium formation. Independent studies also have shown that diacylglycerol can stimulate appressorium formation on normally noninductive surfaces (Thines et al., 1997), indicating that a phospholipase C–inositol trisphosphate pathway may take part in the initial stages of appressorium formation in response to surface cues, such as cutin monomers at the leaf surface. All of the studies to date, however, have been based on the application of inhibitors to wild-type strains of Magnaporthe; our characterization of the  $\Delta cyp1$  mutant offers genetic evidence that calcium signaling regulates appressorium development in Magnaporthe and provides evidence that calcineurin is involved directly.

### Cellular Functions of CYP1 in Magnaporthe

On the basis of the evidence presented here, we have formulated a model to describe the divergent functions of *CYP1* in Magnaporthe. This model is presented in Figure 9. In our model, the *CYP1*-encoded cyclophilin regulates virulence-related functions, including appressorium turgor generation and lipid biosynthesis, and the development of asexual spores. These activities may result from the interaction of the cyclophilin with calcineurin. In yeast, for example, there is some evidence that cyclophilin A interacts with calcineurin even in the absence of CsA (Cardenas et al., 1994), and it has been proposed that the activity of CsA arises by taking advantage of a natural regulatory protein–protein interaction between cyclophilin A and calcineurin. In *C. neoformans*, the *cpa1 cpa2* cyclophilin A double mutants share many phenotypes with calcineurin mutants, also suggesting that cyclophilin A might regulate calcineurin assembly or activity (Wang et al., 2001). The  $\Delta cyp1$  mutant phenotypes are sufficiently diverse, however, that *CYP1* is likely to have a number of other targets and may have a more general effect on gene expression, as discussed above.



**Figure 9.** Model Showing the Predicted Cellular Roles of *CYP1* Cyclophilin in Magnaporthe.

In this model, *CYP1* cyclophilin regulates virulence-associated functions, including penetration peg formation and cellular turgor generation, and also is required for efficient sporulation. Some of these functions may involve an interaction between *CYP1* and calcineurin, perhaps to regulate calcineurin assembly and activity, but there are likely to be a number of other targets with which *CYP1* interacts. The addition of exogenous CsA to Magnaporthe leads to the formation of a *CYP1*–CsA complex, which inactivates calcineurin and prevents calcium/calmodulin-dependent protein phosphatase signaling. Acute sensitivity to CsA indicates that calcineurin is required for appressorium morphogenesis in addition to regulating hyphal growth and development. CAM, calmodulin; CNA, calcineurin A catalytic subunit; CNB, calcineurin B regulatory subunit; CsA, cyclosporin A; *CYP1*, *CYP1*-encoded cyclophilin.

In the second part of our model, the presence of CsA causes the formation of a CYP1-CsA complex that targets calcineurin, thereby preventing its activity in the regulation of appressorium morphogenesis and hyphal development. The functions of *CYP1* are shown separately in our model because we found that appressoria form normally in a  $\Delta cyp1$  mutant, and hyphal development also is not affected by deletion of the cyclophilin. This means that regulation of hyphal development and appressorium formation are not natural functions of cyclophilin in *Magnaporthe*, but inhibition of these developmental processes by CsA does require the presence of *CYP1*. However, there is an omission in our current model that must be addressed. We have predicted, based on its gene organization, that *CYP1* encodes both a mitochondrial and a cytoplasmic form of cyclophilin; however, because our gene replacement deleted the entire *CYP1* open reading frame, we cannot determine whether either or both CYP1 proteins are required for its role in the virulence of *Magnaporthe*. It is likely that CsA sensitivity is mediated through the cytoplasmic CYP1 (Marks, 1996), but we are investigating the role of *CYP1* in virulence by constructing a mutant lacking only the mitochondrial CYP1 cyclophilin (N.J. Talbot and P.V. Balhadère, unpublished data).

The identification of *CYP1* implicates cyclophilins as virulence determinants in phytopathogenic fungi, and preliminary observations of a *Botrytis cinerea* cyclophilin mutant showing attenuated virulence (M.C. Viaud and Y. Brygoo, unpublished data) indicate that cyclophilins may fulfill virulence-related functions in many plant pathogenic fungi. The recent study of cyclophilin function in the human pathogenic fungus *C. neoformans* also suggests that cyclophilins are important determinants of virulence in animal mycoses (Wang et al., 2001). Investigating *CYP1* function may reveal fundamental roles for cyclophilins in fungi and provide insight into the molecular basis of fungal pathogenesis. In addition, the  $\Delta cyp1$  mutant is likely to be an important tool for determining the functional interaction between the calmodulin-dependent, cAMP-dependent, and MAPK signaling pathways responsible for controlling the development of *Magnaporthe* appressoria.

## METHODS

### Fungal Isolates and Culture Conditions

Isolates of *Magnaporthe grisea* used in this study are stored in the laboratory of N.J.T. The fertile wild-type rice (*Oryza sativa*) pathogenic *Magnaporthe* strain Guy11 was used in all studies, and the targeted gene replacement mutants described are isogenic to this strain. The  $\Delta pmk1$  mitogen-activated protein kinase deletion mutant was provided by Dr. Jin Rong Xu (Purdue University, West Lafayette, IN) (Xu and Hamer, 1996). Standard procedures for the culture and maintenance of *Magnaporthe* were performed as described previously (Talbot et al., 1993).

### Nucleic Acid Manipulations

Genomic DNA was extracted from fungal mycelium using a hexadecyltrimethylammonium procedure described previously (Talbot et al., 1993). Gel electrophoresis, restriction enzyme digestion, and DNA gel blot hybridization all were performed using standard procedures (Sambrook et al., 1989). DNA probes were labeled by the random primer method (Feinberg and Vogelstein, 1983) using the Stratagene Prime-It kit, and high-stringency washes were performed as described previously (Talbot et al., 1993). DNA sequence analysis was performed by fluorescent dye terminator cycle sequencing (Amersham-Pharmacia) using an ABI 377 automated sequencer (Perkin-Elmer). DNA/protein sequence databases were searched using the BLAST algorithm (Altschul et al., 1990) at the National Center for Biotechnology Information (Bethesda, MD). RNA was extracted from fungal and rice tissues, and RNA gel blots were prepared as described previously (Dixon et al., 1999). RNA was poly(A) enriched by oligo(dT) affinity chromatography using the Poly(A) Quick mRNA isolation kit (Stratagene).

For differential cDNA screening, radiolabeled cDNA probes were prepared by annealing 1  $\mu$ g of poly(A) RNA to 1  $\mu$ g of oligo(dT) primer and reverse transcribed in the presence of 50  $\mu$ Ci of  $\alpha$ -<sup>32</sup>P-dCTP for 30 min at 37°C using 300 units of Moloney murine leukemia virus reverse transcriptase (Promega). A *Magnaporthe* cDNA library prepared from nitrogen-starved Guy11 mycelium (Lau and Hamer, 1996) was used for differential cDNA screening and identification of a *CYP1* cDNA clone. Genomic clones spanning the *CYP1* locus were selected from a Guy11 genomic library constructed in  $\lambda$ GEM-11 (Talbot et al., 1993). The gapped Blast program (version 2.0; Altschul et al., 1997) was used to compare protein sequences with those contained within databases held at the National Center for Biotechnology Information World Wide Web site (<http://www.ncbi.nlm.nih.gov/>). Multiple sequence alignments were performed using the Multalign program (<http://dot.imgen.bcm.tmc.edu:9331/multi-align/multi-align.html>) (Thompson et al., 1994).

For reverse transcriptase-polymerase chain reaction (PCR) experiments, two forward primers were designed that were specific to the longer form of *CYP1*, and one was designed that was specific to the shorter form of *CYP1*. These were MC5.1 at position 544 with sequence 5'-TCTTCTCGACCCCTCGTTC-3', MC5.2 at position 630 with sequence 5'-CACCGAAATCTCCCATCAAC-3', and CC5.1 at position 819 with sequence 5'-TTCGACATTTCTTCAACG-3'. One reverse primer (CC3.1) at position 1417 of the *CYP1* sequence was used with sequence 5'-TGGGGGTACCGCTGCTGGAG-3'. Total RNA was extracted from each *Magnaporthe* strain and reverse transcribed to cDNA using an oligo(dT) primer and Moloney murine leukemia virus reverse transcriptase for 1 hr at 37°C under conditions recommended by the enzyme manufacturer (Promega). Then, PCR was performed using 10 pmol of each primer for 30 cycles of 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C. Amplifications from genomic DNA templates were performed under identical conditions. Control amplifications of a 200-bp fragment of the *Magnaporthe* actin gene were performed from DNA and cDNA templates using primers ACT5 (5'-TGGCACCACACCTTCTACAA-3') and ACT32 (5'-CGGAGTCGAGCAGATACCA-3').

Phylograms were generated using maximum parsimony analysis with the branch-swapping and nearest-neighbor interchange options in PAUP\* version 4.0s (Swofford, 2000). The consistency of the phylogenetic trees was determined using the heuristic search program, and branch strengths were tested by 100 repetitions of the bootstrap algorithm with the branch-swapping and nearest-neighbor interchange options (Swofford, 2000). Protein subcellular localization was

predicted using PSortII (Nakai and Kanehisa, 1992) at the Okasaki server (Okasaki, Japan).

### Construction of the Gene Replacement Vector pΔCYP1 and Complementation Analysis

The targeted gene replacement vector pΔCYP1 was constructed by first cloning a 4.3-kb XhoI genomic fragment into pGEM-11Z (Promega) to create pCYP1X. This vector then was digested with PstI to delete a 1.1-kb PstI fragment containing the *CYP1* open reading frame, and a selectable marker gene cassette bestowing hygromycin B resistance was ligated in its place. PstI sites were added to a 1.4-kb DNA fragment containing the hygromycin phosphotransferase gene from *Escherichia coli* under the control of the *Aspergillus nidulans* *trpC* promoter (Carroll et al., 1994) by PCR with the PHYG primer 5'-CGCCTGCAGGAATTCGTCGACGTTAAC-3' and the proofreading thermostable enzyme *Pfu* polymerase (Promega). The resulting PCR amplicon was digested with PstI and ligated to the digested pCYP1X vector to make pΔCYP1. For complementation analysis, the 1.1-kb PstI fragment and a 4.3-kb XhoI fragment, which span the *CYP1* gene, were cloned individually into pCB1265, which carries the bar gene for bialaphos resistance, to create pMVN57P and pMVN57X, respectively.

### Fungal Transformation

Protoplasts were prepared from Magnaporthe as described previously (Talbot et al., 1993). DNA-mediated transformation was performed using 2 μg of pΔCYP1 that was first linearized with BamHI. Transformed protoplasts were plated in molten osmotically stabilized complete medium (CM) agar (Talbot et al., 1993), and an overlay of CM agar containing 250 μg/mL hygromycin B (Calbiochem) or 35 μg/mL bialaphos (Aventis Crop Sciences, Ongar, UK) was added after 18 hr. Transformants were selected after 6 to 8 days at 24°C and screened for the gene replacement event or *CYP1* complementation by DNA gel blot analysis.

### Rice Blast Pathogenicity Assays

Rice blast infections were performed using 12-day-old rice seedlings of the susceptible cv CO-39. Seedlings were sprayed with conidial suspensions at concentrations of 10<sup>4</sup> and 10<sup>5</sup> conidia/mL in 0.1% gelatin (Dixon et al., 1999). The percentage of blast-infected leaves was determined after 5 days, disease lesion densities were recorded from 10 randomly selected leaves, and means and standard deviations were determined. Pathogenicity assays were performed three times using 50 plants per assay.

### Assays for Infection-Related Morphogenesis and the Activity of Cyclosporin A

Appressorium development by Magnaporthe was observed on plastic cover slips as described previously (Dixon et al., 1999; Thines et al., 2000). Conidia were harvested from 12-day-old plate cultures of Magnaporthe, and a 50-μL drop of conidial suspension, at a concentration of 10<sup>5</sup> conidia/mL, was placed on the surface of a plastic cover slip (PGC Scientific, Frederick, MD) and incubated in a humid environment at 24°C. The formation of abundant appressoria was

stimulated under these conditions by surface hydrophobicity (Hamer et al., 1988; Lee and Dean, 1994), and the frequency of appressorium formation was determined by counting the number of appressoria that had developed from 100 conidia after 20 hr (Talbot et al., 1993).

Appressorium turgor was estimated using an adaptation of the incipient cytorrhysis technique (Howard et al., 1991; de Jong et al., 1997). Appressoria were allowed to form on plastic cover slips, and the water surrounding them was replaced carefully with aqueous glycerol solutions varying from 0.5 to 5.0 M. The number of appressoria that had collapsed after 15 min was recorded. The effect of the immunosuppressive drug cyclosporin A (CsA) on the infection-related morphogenesis of Magnaporthe was tested by adding 0, 1, 5, 10, 20, and 100 μg/mL CsA (Sigma) in 1% DMSO to conidia before germination and the elaboration of appressoria.

All experiments were performed at least three times using three replicate cover slips per experiment. The effect of CsA on the vegetative growth of Magnaporthe was tested in two different ways, as shown in Figures 6 and 7. Vegetative growth from conidia was determined first by taking filter paper discs (0.5 cm in diameter) inoculated with 20 μL of 0, 1, 10, 100, and 1000 μg/mL CsA in 1% DMSO. A suspension of conidia containing ~3 × 10<sup>3</sup> conidia was spread on each CM agar plate (Talbot et al., 1993), and the CsA filters were placed on the agar surface. The diameter of the inhibition growth zone was measured after 3 days. Vegetative growth also was assessed by taking a plug of mycelium and placing it on the center of a CM agar plate inoculated with 100 μg/mL CsA. Plates were held at 24°C for 5 days. All CsA inhibition experiments were repeated three times with three replicates per experiment.

### Accession Number

The GenBank accession number for CYP1 is AF293848.

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