The Plant Cell, Vol. 8, 771–781, May 1996

RESEARCH ARTICLE

Regulatory Genes Controlling *MPG1* Expression and Pathogenicity in the Rice Blast Fungus *Magnaporthe grisea*

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***MPG1*, a pathogenicity gene of the rice blast fungus *Magnaporthe grisea*, is expressed during pathogenesis and in axenic culture during nitrogen or glucose limitation. We initiated a search for regulatory mutations that would impair nitrogen metabolism, *MPG1* gene expression, and pathogenicity. First, we developed a pair of laboratory strains that were highly fertile and pathogenic toward barley. Using a combinatorial genetic screen, we identified mutants that failed to utilize a wide range of nitrogen sources (e.g., nitrate or amino acids) and then tested the effect of these mutations on pathogenicity. We identified five mutants and designated them *Nr*⁻ (for nitrogen regulation defective). We show that two of these mutations define two genes, designated *NPR1* and *NPR2* (for nitrogen pathogenicity regulation), that are essential for pathogenicity and the utilization of many nitrogen sources. These genes are nonallelic to the major nitrogen regulatory gene in *M. grisea* and are required for expression of the pathogenicity gene *MPG1*. We propose that *NPR1* and *NPR2* are major regulators of pathogenicity in *M. grisea* and may be novel regulators of nitrogen metabolism in fungi.**

INTRODUCTION

Although the vast majority of fungi exist as benign saprotrophs, others are able to exploit wounded or debilitated hosts. Only a minority of fungi are devastating pathogens of healthy eukaryotic hosts and can exploit the host environment by a variety of specific adaptations. The rice blast fungus *Magnaporthe grisea* provides a striking example of adaptations for pathogenicity and can be easily manipulated for both genetic and biochemical studies of phytopathogenicity (Valent, 1990).

In *M. grisea*, pathogenicity is a complex trait and involves the formation of specialized cell types, including spores, appressoria, and infectious hyphae (reviewed in Talbot, 1995). A number of approaches have been used to identify and study genes controlling pathogenicity. For example, several genes involved in spore formation have been found to have pleiotropic effects on pathogenesis (Hamer et al., 1989; Hamer and Givan, 1990; Shi and Leung, 1994). Mutations in genes required for melanin biosynthesis also affect pathogenicity (Chumley and Valent, 1990). Insertional mutagenesis with transforming plasmids also has been used to identify and tag genes involved in pathogenesis (J. Sweigard, A. Carroll, F. Chumley, and B. Valent, unpublished data; G. Lau and J.E. Hamer, unpublished data). Recently, differential cDNA screening has been used to isolate *M. grisea* genes that are highly expressed in the plant (Talbot et al., 1993). One highly expressed gene, designated *MPG1*, encodes a small hydrophobic protein that belongs to a family of fungal proteins called hydrophobins (reviewed in Templeton et al., 1994). Deletion of the *MPG1* gene causes a reduction in pathogenicity.

MPG1 is highly expressed during early and late stages of pathogenic growth. Its expression is suppressed during axenic culture in rich media, but the steady level of its mRNA increases dramatically during nitrogen or carbon limitation (Talbot et al., 1993). Similarly, two putative pathogenicity genes expressed in the plant as well as the avirulence gene *AVR9* from the tomato wilt pathogen *Cladosporium fulvum* also are induced during starvation for nitrogen (van den Ackerveken et al., 1993, 1994). Nutrient deprivation elevates the expression of virulence genes in several phytopathogenic bacteria (Rahme et al., 1992; Schulte and Bonas, 1992; Wei et al., 1992), and the invasive growth state of *Saccharomyces cerevisiae* requires growth on poor or limiting nitrogen sources (Gimeno et al., 1992). Thus, although regulatory mechanisms controlling pathogenic growth are largely unknown (especially in fungi), studies in several systems implicate a regulatory pathway involving starvation for preferred nitrogen or carbon sources. One regulatory mechanism may involve nitrogen regulation (NR).

NR has been well studied in Gram-negative bacteria and in the filamentous fungi *Aspergillus nidulans* and *Neurospora crassa*. In these microbes, preferred nitrogen sources, such as glutamine or ammonium, are utilized preferentially over amino acids, nucleotides, or more complex nitrogen sources. This

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is accomplished by the selective expression of genes required for exogenous nitrogen source utilization only in the absence of glutamine or ammonia (Crawford and Arst, 1993). In *Escherichia coli* and *Salmonella typhimurium*, NR is a complex process involving a number of regulatory genes, such as *rpoN/ntrA*, *glnG/ntrC*, or *glnL/ntrB* (reviewed in Magasanik and Neidhardht, 1987). Homologs of these genes in *Pseudomonas* spp have been shown to regulate genes involved in plant pathogenesis (Schulte and Bonas, 1992).

In filamentous fungi, nitrogen utilization pathways are activated by wide-domain regulators (Arst and Cove, 1973). In *A. nidulans* and *N. crassa*, these wide-domain regulators are members of the GATA family of DNA binding proteins and have been shown to be transcriptional activators encoded by the *areA* and *nit-2* genes, respectively (Caddick et al., 1986; Fu and Marzluf, 1990). These genes are functional homologs of each other and differentially activate hundreds of genes involved in nitrogen assimilation. Null mutations in these regulatory genes render strains unable to utilize a wide variety of nitrogen sources, with the exception of ammonium or glutamine.

Our study of *MPG1* led us to hypothesize that mutations in a wide-domain regulator of NR might render *M. grisea* nonpathogenic due to an inability to derepress a subset of genes required for pathogenicity. A functional homolog of *areA/nit-2* has been identified recently in *M. grisea* and designated *NUT1* (for nitrogen utilization). Surprisingly, *NUT1* was shown to be dispensable for pathogenicity (Froeliger and Carpenter, 1996). Here, we describe a successful genetic screen to identify wide-domain regulators of NR and pathogenicity. We identified two genes, designated *NPR1* and *NPR2* (for nitrogen pathogenicity regulation genes 1 and 2), that when mutated cause an inability to utilize a wide range of nitrogen sources and a dramatic loss of pathogenicity. We show that $Npr1^-$ and $Npr2^-$ mutants grow poorly on a wide range of nitrogen sources and that *NPR2* is required for induction of nitrate reductase. *NPR1* and *NPR2* are required for expression of the *MPG1* hydrophobin. Thus, in addition to affecting NR, *NPR1* and *NPR2* appear to be *trans*-acting positive effectors of genes involved in pathogenicity. These studies demonstrate a link between metabolic gene regulation and pathogenicity in the rice blast fungus.

RESULTS

Breeding of the Highly Fertile Laboratory Strains of *M. grisea* Pathogenic on Barley

M. grisea isolates can infect a wide range of monocot hosts, although the host range of individual strains is very narrow. We recently found that several host-specific forms of *M. grisea*, including several rice pathogens, can infect the barley cultivar Golden Promise. Because of the ease of cultivation of barley, the wide leaf area for observing symptoms, and the rapid onset of disease, we generated highly fertile laboratory strains that were pathogenic toward barley, using the breeding scheme shown in Figure 1A. The starting strain G-22, first described as WGG-FA40 (Yaegashi and Asaga et al., 1981), is a finger millet pathogen, and G-17, described as K76-79 (Heath et al., 1990), is a weeping lovegrass pathogen. The resulting strains 35-R-24 and 35-R-56 were selected on the basis of high fertility, culture morphology, and pathogenicity toward barley. Although these strains do not infect rice, we have found that the superficial cytological aspects of *M. grisea* infections of barley and rice are similar. A complete description of symptom development on barley will be presented elsewhere (G. Lau, M. Murcia, and J.E. Hamer, manuscript in preparation).

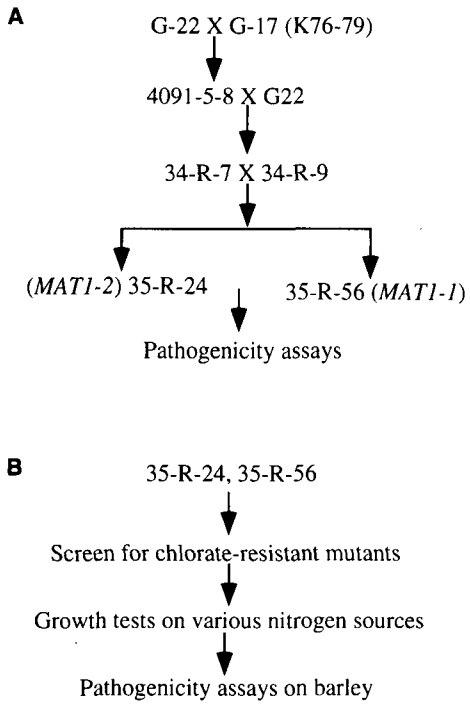

Figure 1. Pedigree of the Crosses Leading to Strains 35-R-24 and 35-R-56.

(A) Strains G-22 and G-17 are wild isolates collected by H. Yaegashi (Tohoko National Agriculture Experimental Station, Omagari, Japan), and this cross is described by Valent et al. (1986). Progeny from the cross of 4091-5-8 × G-22 were tested for pathogenicity toward barley, fertility, and culture morphology. Two fertile progeny of opposite mating type were crossed (34-R-7 × 34-R-9), and progeny were rescreened. Two progeny of opposite mating type from this cross, 35-R-24 and 35-R-56, were designated as wild type for subsequent mutagenesis.
(B) Combined screen for nitrogen utilization and pathogenicity-defective mutants. Chlorate-resistant mutants were screened for their ability to utilize various nitrogen sources and pathogenicity toward barley.

Isolation and Characterization of Chlorate-Resistant Mutants

Chlorate resistance can be used in a variety of systems to identify genes involved in nitrogen assimilation, and the basis for chlorate toxicity in fungi has been described (Cove, 1976). Approximately 380 chlorate-resistant mutants were isolated from *M. grisea* strains 35-R-24 and 35-R-56 (see Methods). Mutants were categorized by growth tests, using various nitrogen sources and pathogenicity assays (Table 1). Growth phenotypes are described by using genetic nomenclature used for *A. nidulans* (Arst and Cove, 1973). Figure 1B outlines the screen for mutants defective in nitrogen regulation and pathogenicity.

Of 353 mutants screened, 250 failed to grow on minimal medium supplemented with nitrate or adenine–hypoxanthine as a sole nitrogen source. This class of mutants, designated Cnx$^-$, presumably represents mutations in genes that are involved in the synthesis of the molybdenum cofactor for nitrate reductase and xanthine dehydrogenase. Fifty mutants exhibited no observable defect in nitrogen metabolism, except that they were chlorate resistant. This class of mutants, designated CrnA$^-$, presumably represents mutations in the nitrate transporter. Thirty mutants showed loss of growth on nitrate and nitrite. This class of mutants, designated NiiA$^-$/NirA$^-$, is presumably due to mutations in nitrite reductase (NiiA$^-$) or a pathway-specific transcription activator of both the nitrate and nitrite reductases (NirA$^-$). Eighteen mutants were characterized by their inability to grow on nitrate. Designated NiaD$^-$, they presumably carry mutations in the structural gene for nitrate reductase. A subset from each of these mutant classes was tested for pathogenicity toward the barley cultivar Golden Promise. All of the tested strains were pathogenic (data not shown), and no further genetic analysis was undertaken.

Table 1. Classes of Spontaneous Chlorate-Resistant Mutants in *M. grisea*

Classes[a]	No. of Mutants	Growth Phenotypes	Pathogenicity[b]
Cnx$^-$	250	Poor growth on nitrate and adenine	+
CrnA$^-$	50	Chlorate resistant	+
NiiA$^-$/NirA$^-$	30	Poor growth on nitrate and nitrite	+
NiaD$^-$	18	Poor growth on nitrate	+
Nr$^-$	4	Glutamine-dependent growth[c]	+
Npr$^-$	2	Glutamine-dependent growth[c]	–

[a] Nomenclature is based on that of *A. nidulans* (see text), except for the Nut$^-$ and Npr$^-$ mutants.
[b] (+), fully pathogenic; (–), reduced pathogenicity. Infection assays were performed on barley, as described in Methods.
[c] Strains grow well on glutamine but poorly on other nitrogen sources.

Mutations at *NPR* Genes Cause a Defect in Nitrogen Metabolism and Pathogenicity

In addition to these fully pathogenic *M. grisea* chlorate-resistant mutants, we identified five mutants that showed an inability to utilize a wide range of nitrogen sources; these were provisionally designated Nr$^-$, for nitrogen regulation defective. These mutants, along with an Nr$^-$ mutant, Guy11397C113, derived from a rice pathogen strain Guy11 (obtained from M.H. Lebrun, University of Paris–Sud, Orsay, France), were tested for pathogenicity toward barley and rice, respectively. Surprisingly, only two out of these six Nr$^-$ mutants were nonpathogenic. Both nonpathogenic Nr$^-$ mutants were obtained from parent strain 35-R-24. This phenotypic class was designated Npr$^-$ for nitrogen and pathogenicity regulation defective (Table 1).

Sample leaves from pathogenicity assays of parents 35-R-24 and 35-R-56 and Npr$^-$ mutant strains CI60 and CI105 are shown in Figure 2. Strains 35-R-24 (Figure 2B) and 35-R-56 (Figure 2C) are highly virulent and produce large, spreading lesions. In contrast, leaves infected with CI60 and CI105 showed only a few minute lesions (Figures 2D and 2E, respectively). Guy11397C113, CI60, and CI105 were found to be defective in utilization of a variety of nitrogen sources, including nitrate, nitrite, adenine, arginine, glycine, histidine, leucine, lysine, and phenylalanine. The growth defect in Guy11397C113 (Nr$^-$) was more severe than it was in CI60 and CI105 (Npr$^-$) for all amino acids tested. Npr$^-$ mutants typically give sparse, spidery mycelial growth on amino acid plates. However, all three strains were able to grow on glutamine, as expected. Growth on glutamine, nitrate, and histidine is shown in Figure 3. We conclude that strains CI60 and CI105 contain mutations that impair nitrogen source utilization and pathogenicity.

To test whether the reduction in pathogenicity (Pth$^-$) was due to the same mutation that conferred chlorate resistance (ClO_3^r), CI60 and CI105 were crossed with wild-type strain 35-R-56 that is chlorate sensitive (ClO_3^s, Pth$^+$). Progeny that were chlorate resistant were dramatically reduced in pathogenicity, whereas all the progeny that were sensitive to chlorate were fully pathogenic (data not shown). Segregation ratios were 18:16 (Npr$^+$:Npr$^-$) for CI60 and 21:18 for CI105. No recombinant progeny (ClO_3^s, Pth$^-$ or ClO_3^r, Pth$^+$) were recovered from either cross.

Chlorate-resistant progeny of CI60 and CI105 were crossed to test whether they carry mutations in the same *NPR* gene. In two separate crosses (crosses 62 and 63), ~25% of the progeny were wild-type recombinants, as demonstrated by their ability to utilize nitrate (Table 2). These results indicate that CI60 carries a mutation that is different and unlinked to the mutation in CI105. Approximately 25% of these segregants should have represented double mutants. Although double

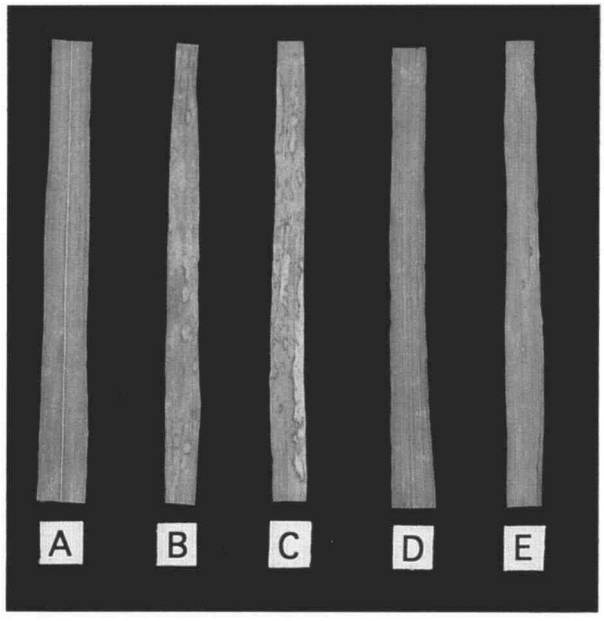

Figure 2. Pathogenicity Assays of the Wild Type and Npr$^-$ Mutants.

Pathogenicity was performed by spray inoculation, as described previously (Talbot et al., 1993), using the barley cultivar Golden Promise. Typical leaves are shown.

(A) Healthy, uninfected barley leaf.

(B) and **(C)** Barley leaves infected with wild-type strains 35-R-24 and 35-R-56, respectively.

(D) and **(E)** Barley leaves infected with Npr$^-$ mutants Cl60 and Cl105, respectively.

mutants were not genetically characterized, the observation that the remaining 75% of the segregants were nitrate nonutilizing suggests that double mutants also are impaired in nitrogen metabolism.

NPR1 and *NPR2* Are Unlinked from *NUT1*

To confirm that Cl60 (*npr1-1*) and Cl105 (*npr2-1*) carry mutations at loci different from *NUT1*, we performed allelism tests with Cl60, Cl105, and a known *NUT1* deletion mutant, Guy11nut1-4. This strain contains a gene disruption engineered into the *NUT1* locus, using a hygromycin resistance gene (Froeliger and Carpenter, 1996). The Guy11nut1-4 strain shows the expected phenotype of an inability to utilize a wide range of nitrogen sources. The *NUT1* gene also complements null mutations in the *A. nidulans areA* gene (Froeliger and Carpenter, 1996). To follow segregation patterns, we took advantage of the fact that Cl60 and Cl105 are ClO_3^r but hygromycin B sensitive (Hyg^s). In contrast, Guy11nut1-4 is hygromycin B resistant (Hyg^r) and much more sensitive to chlorate. We followed the segregation patterns among the progeny of Npr$^-$ × Nut$^-$ crosses and looked for the presence of two classes of recombinants carrying wild-type (Hyg^s, ClO_3^s) and double-mutant (Hyg^r, ClO_3^r) phenotypes. The results of the crosses are presented in Table 3. In both cases, segregants of the recombinant classes were recovered. The segregation ratio that was not 1:1:1:1 could have been the result of the apparent low viability of recovered ascospores and can be attributed to the lack of isogenicity between 35-R-24 and Guy11.

nit-2 Complements a Nut1$^-$ Mutation but Not Npr1$^-$ or Npr2$^-$ Mutations

To test whether *npr1-1* or *npr2-1* mutations reside in another *areA/nit-2/NUT1* homolog, we transformed Npr$^-$ strains with pNIT2, a plasmid containing the *nit-2* gene of *N. crassa* (Fu and Marzluf, 1987). The *nit-2* gene has been shown to function in heterologous ascomycete hosts (Davis and Hynes, 1987). We introduced the *nit-2* gene of *N. crassa* into both Npr$^-$ (Cl60 and Cl105) and Nr$^-$ (Guy11397Cl13) mutants. Although allelism was not tested between the Nr$^-$ mutation in Guy11397Cl13 and the Guy11nut1-4 strain, phenotypic analy-

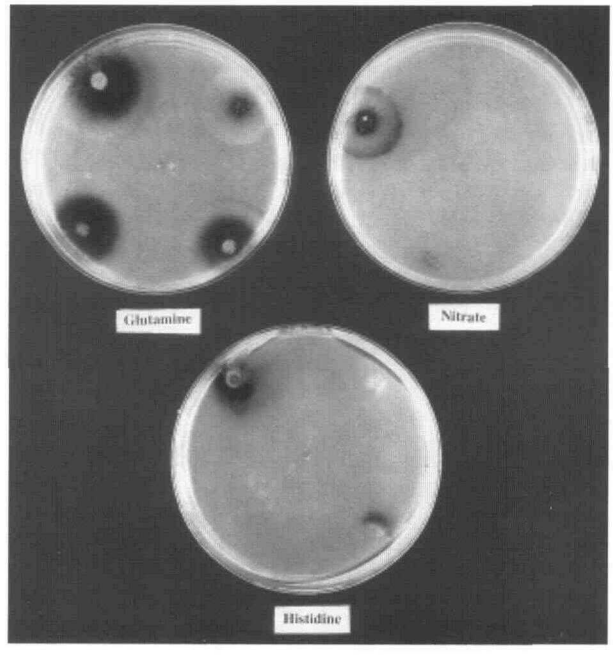

Figure 3. Growth Tests for Nitrogen Utilization by the Wild Type and Npr$^-$ Mutants.

Tanaka minimal medium (Tanaka, 1965) was supplemented with 6 mM individual nitrogen sources. All the strains were inoculated in the same order for each plate. The top left of each plate was inoculated with wild-type strain 35-R-24. The top right of each plate was inoculated with Nr$^-$ mutant Guy11397C113. The bottom left of each plate was inoculated with Npr1$^-$ strain Cl60. The bottom right of each plate was inoculated with Npr2$^-$ strain Cl105.

Table 2. Segregation Analysis of the Crosses between Npr1⁻ and Npr2⁻ Strains

Cross	Parents Npr1⁻ × Npr2⁻	Random Ascospore Progeny Number	No. of Wild-Type Recombinants[a]
62	43-R-20 × 44-R-22	111	25
63	43-R-35 × 44-R-17	57	14

[a] The number of wild-type recombinants is based on the number of progeny capable of utilizing nitrate as sole nitrogen source.

sis strongly suggested that the Nr⁻ mutation in strain Guy11397Cl13 resides in the *NUT1* gene (G. Lau and J.E. Hamer, unpublished data). *M. grisea* mutant strains were transformed with pNIT2, and transformants were selected directly on nitrate minimal medium, taking advantage of the inability of both Nr⁻ and Npr⁻ mutants to utilize nitrate. Figure 4 shows pNIT2 integration events and growth tests of transformants. Guy11397Cl13 was successfully transformed with pNIT2, judging by the ability of transformants to utilize nitrate (Figure 4B) and the integration of pNIT2 DNA (Figure 4A). These results further suggest that Guy11397Cl13 carries a mutation at *NUT1*. However, we failed to recover any transformants of Cl60 and Cl105 that were able to grow on nitrate.

In a second strategy, we performed cotransformations of pNIT2 and a hygromycin B resistance plasmid. The transformants were selected on 200 mg/mL hygromycin B and screened for growth on nitrate minimal medium. DNA gel blot analysis of two Cl60 and four Cl105 hygromycin-resistant transformants showed integration of pNIT2 DNA (Figure 4A, lanes 4 and 5, and lanes 7 to 10, respectively). However, these transformants failed to grow on nitrate (Figure 4B) and were still nonpathogenic toward barley (data not shown). These results strongly suggest that Npr1⁻ and Npr2⁻ are not functionally complemented or suppressed by the *N. crassa nit-2* gene.

NUT1 and *NPR2* Are Required for the Induction of Nitrate Reductase

Growth tests and genetic analysis suggest that *NPR1* and *NPR2* are novel wide-domain regulators of NR. *NUT1* also has been defined as a major regulator of NR in *M. grisea*. However, there have been no studies of nitrogen-regulated gene expression in *M. grisea*. We examined the steady state mRNA levels of the nitrate reductase gene (*NIAD*) in Nr⁻, Npr1⁻, and Npr2⁻ strains. Although studies with the *M. grisea NIAD* gene had not been performed previously, studies with other filamentous fungi have shown that *NIAD* is induced by nitrate and repressed by glutamine and ammonium (Arst and Cove, 1973; Dunn-Coleman et al., 1981).

RNA was extracted from cultures grown under noninducing (complete medium supplemented with 10 mM ammonium sulfate and 2% yeast extract) and inducing (minimal medium supplemented with nitrate) conditions. The steady state level of *NIAD* mRNA is shown in Figures 5A and 5B. RNA gel blot analysis was performed using gene-specific probes (see Methods). A nitrate reductase transcript was undetectable under the noninducing condition in all the strains tested (Figure 5B, lanes 1 to 4). Under the inducing condition, a highly elevated *NIAD* RNA level was detected in wild-type strain 35-R-24 (Figure 5B, lane 5). No induction was detected in the Nr⁻ strain (Figure 5B, lane 6). These results are consistent with typical results obtained for nitrogen catabolite regulation of the nitrate reductase in *A. nidulans* (Arst and Cove, 1973). We conclude that *NUT1* is a positive regulator of nitrate reductase in *M. grisea* and that some aspects of the nitrogen regulatory circuitry described for *A. nidulans* are conserved in *M. grisea*.

Figure 5B (lane 8) shows that a mutation in *NPR2* also prevents induction of *NIAD* mRNA. These results are consistent with growth tests that show that *npr2-1* strains fail to grow well on nitrate as a sole nitrogen source. Surprisingly, an elevated level of *NIAD* mRNA was detected in the *npr1-1* mutant strain, CL60, when grown under inducing conditions (Figure 5B, lane 7). The level of derepression was comparable with that of wild-type strain 35-R-24 (Figure 5B, lane 5). This result was unanticipated because strain *npr1-1* grows poorly on nitrate (see Figure 3). Because metabolism of nitrate requires several gene products, we think it is likely that *NPR1* may affect the expression of components other than *NIAD* in the nitrate assimilation pathway. We conclude that *NPR2* and *NUT1* are required for induction of the nitrate reductase gene.

NUT1, *NPR1*, and *NPR2* Are Required for High-Level Expression of *MPG1* during Nitrogen Limitation

The rationale for our genetic screen was to identify regulators of pathogenicity gene expression. Figures 6A and 6B show the steady state level of the pathogenicity gene *MPG1* RNA levels in various mutant backgrounds during growth under nutrient-rich and nitrogen-limited growth conditions. Growth

Table 3. Segregation Analysis between a *NUT1* Mutant (Guy11nut1-4) and *NPR* Mutants (Cl60 and Cl105)

Cross	Parents	Parental Progeny		Recombinant Progeny	
		ClO_3^r Hyg^s	ClO_3^s Hyg^r	ClO_3^s Hyg^s	ClO_3^r Hyg^r
56	Guy11nut1-4 × Cl60	13	5	30	22
57	Guy11nut1-4 × Cl105	4	2	6	7

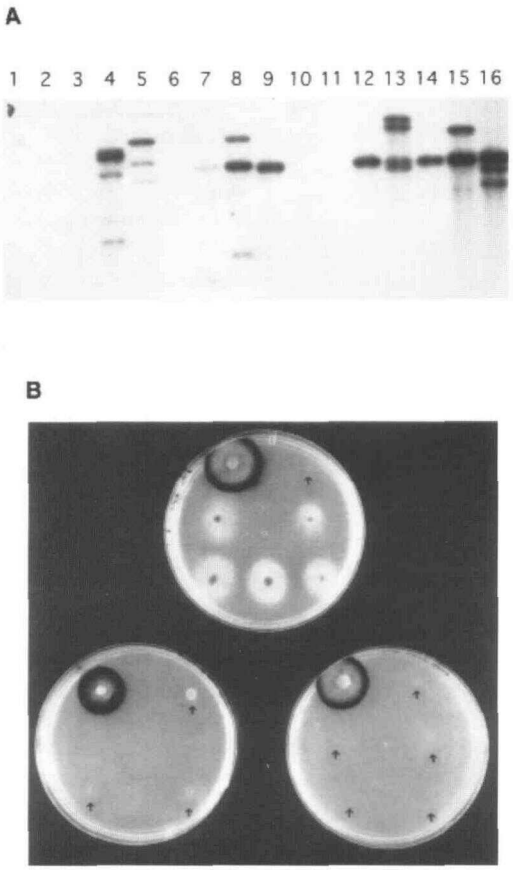

Figure 4. Rescue of the Nr⁻ Mutant but Not Npr⁻ Mutants by pNIT2 Plasmids of *N. crassa*.

(A) DNA gel blot analysis of the pNIT2 transformants. Genomic DNA was extracted as described previously (Talbot et al., 1993). DNA was digested with EcoRI and probed with a 6-kb EcoRI fragment from pNIT2 (Fu and Marzluf, 1987). Lane 1 contains 35-R-24; lane 2, 35-R-56; lane 3, CI60; lanes 4 and 5, pNIT2 transformants Tm103 and Tm155 of CI60, respectively; lane 6, CI105; lanes 7 to 10, pNIT2 transformants Tm1, Tm4, Tm6, and Tm7 of CI105, respectively; lane 11, Guy11397CI13; lanes 12 to 16, pNIT2 transformants Tm1 to Tm5 of Guy11397CI13, respectively. Lane 10 is somewhat underloaded, and longer exposures reveal hybridization to the pNIT2 probe.

(B) Growth test of pNIT2 transformants of Nr⁻ mutant Guy11397CI13 (top plate) and Npr⁻ mutants CI60 (bottom left) and CI105 (bottom right) on nitrate minimal medium. The upper left corner of each plate was inoculated with wild-type strain 35-R-24 as a positive control. The upper right of each plate was inoculated with each respective pNIT2 recipient (mutant strains). The lower part of each plate was inoculated with pNIT2 transformants as described in **(A)**. Arrows indicate the positions of the inoculum.

in rich media resulted in a low level of *MPG1* transcript in both the wild type and Nr⁻, and in Npr⁻ strains (Figure 6B, lanes 1 and 2). The basal level of *MPG1* mRNA in Figure 6 is slightly higher than we and others previously found in strain Guy11 (Talbot et al., 1993; J. Beckerman and D.J. Ebbole, submitted manuscript). Although we do not know the basis for this difference, we suspect different genetic backgrounds or subtle differences in growth conditions may affect the basal level of *MPG1* mRNA. In addition, we and others (J. Beckerman and D.J. Ebbole, personal communication) have found that nitrogen catabolite derepression occurs rapidly in *M. grisea*, and maintaining repressed growth conditions requires growth in 2% yeast extract for short periods.

When grown under conditions of nitrogen limitation, *MPG1* mRNA levels increased dramatically in the wild-type strain but less so in the Nr⁻ mutant (Figure 6B, lanes 5 and 6). These results demonstrate that the *NUT1* gene is responsible, in part, for the high-level expression of *MPG1* during nitrogen starvation. The protein sequence of the *NUT1* gene identifies it as a GATA box binding transcription factor (Froeliger and Carpenter, 1996). Consistent with this observation and our RNA blot experiments, the upstream region of the *MPG1* gene contains several consensus GATA sequences (positions −54 to −49, −464 to 458, and −499 to −494). We conclude that high levels of *MPG1* expression require *NUT1*. The basal levels of *MPG1* expression may be sufficient for pathogenicity because *NUT1* is dispensable for pathogenicity. Alternatively, additional

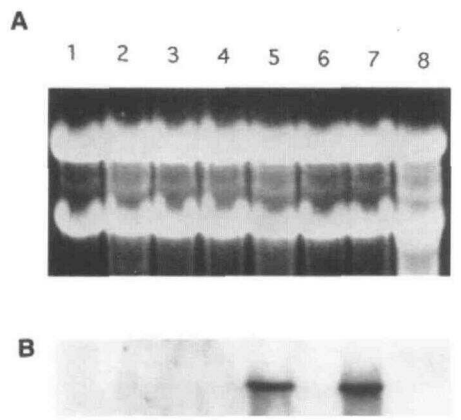

Figure 5. *NPR2* and *NUT1* but Not *NPR1* Are Required for the Expression of Nitrate Reductase during Induction with Nitrate.

RNA samples were fractionated on a 1.2% formaldehyde–agarose gel and transferred to a nylon membrane, as described in Methods. Approximately 30 μg of RNA was loaded into each lane in the following order: lanes 1 to 4 contain total RNA samples extracted from cultures grown in complete medium supplemented with 10 mM ammonium sulfate and 2% yeast extract; lanes 5 to 8 contain total RNA samples extracted from cultures grown in nitrate-containing medium. Lanes 1 and 5 contain wild-type strain 35-R-24; lanes 2 and 6, Nr⁻ mutant Guy11397CI13; lanes 3 and 7, Npr1⁻ mutant CI60; lanes 4 and 8, Npr2⁻ mutant CI105.

(A) Ethidium bromide–stained gel.

(B) RNA gel blot analysis of the gel shown in **(A)**. The blot was hybridized to a NIAD-containing plasmid pCB848.

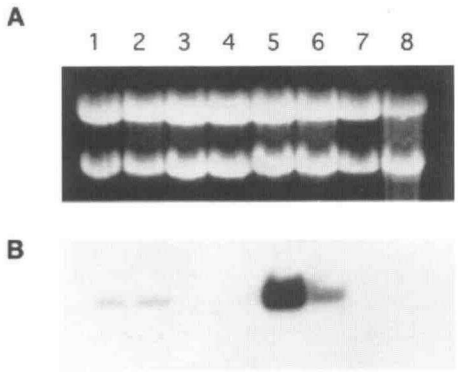

Figure 6. *NUT1*, *NPR1*, and *NPR2* Are Required for the Full Derepression of the Pathogenicity Gene *MPG1* during Nitrogen Starvation.

RNA samples were fractionated on a formaldehyde–agarose gel and transferred to a nylon membrane, as described in Methods. Approximately 30 μg of RNA was loaded into each lane. Lanes 1 to 4 contain RNA samples extracted from cultures grown in complete medium. Lanes 5 to 8 contain RNA samples extracted from cultures grown in nitrogen-starved media. Lanes 1 and 5, wild-type strain 35-R-24; lanes 2 and 6, Nr⁻ mutant Guy11397Cl13; lanes 3 and 7, Npr1⁻ mutant Cl60; lanes 4 and 8, Npr2⁻ mutant Cl105.

(A) Ethidium bromide–stained gel.

(B) RNA gel blot analysis of the gel shown in **(A)**. The blot was hybridized with the 2.7-kb XbaI-BamHI fragment containing the *MPG1* gene from plasmid pNJT15GXB (Talbot et al., 1993).

genetic controls may affect *MPG1* expression during pathogenic growth.

In contrast with the above results, the basal level of the *MPG1* transcript is dramatically reduced in both *npr1-1* and *npr2-1* mutants (Figure 6B, lanes 3 and 4, respectively). In both Npr⁻ mutant strains, high levels of *MPG1* transcript were not detected during nitrogen limitation (Figure 6B, lanes 7 and 8, respectively). We conclude that *NPR1* and *NPR2* are required for *MPG1* expression. These results are consistent with the observation that *npr1-1* and *npr2-1* strains are defective in pathogenicity.

NPR1 and *NPR2* but Not *NUT1* Are Required for the Full Expression of *MPG1* during Carbon Limitation

Talbot et al. (1993) demonstrated that *MPG1* also was induced under conditions of carbon limitation. Figures 7A and 7B show *MPG1* mRNA levels during limitation for carbon in wild-type and mutant strains. The steady state *MPG1* mRNA level during carbon limitation is unaffected by the *nut1* mutation when compared with the wild-type strain 35-R-24 (Figure 7B, lanes 1 and 2). This result is expected given the proposed role of *NUT1* in nitrogen regulation. Surprisingly, the *MPG1* message was virtually undetectable in both Npr⁻ mutants (Figure 7B, lanes 3 and 4). These results suggest that both *NPR1* and *NPR2* may have broader regulatory functions than *NUT1* because they are required for *MPG1* transcript accumulation during both carbon and nitrogen limitation.

NPR1 and *NPR2* Are Not Required for Expression of *CPKA*

Currently, there are few pathogenicity genes that have been identified in *M. grisea*. Recent studies have demonstrated the importance of cAMP signaling in pathogenesis (Mitchell and Dean, 1995; J. Sweigard, personal communication). We used a gene-specific probe for a recently cloned *M. grisea* cAMP-dependent protein kinase gene (*CPKA*; see Methods) to examine the steady state mRNA levels in *npr1* and *npr2* mutants. Figures 8A and 8B show that limitation for nitrogen (or carbon; data not shown) had minor effects on *CPKA* mRNA levels. Consistent with this finding, *CPKA* mRNA levels were slightly reduced in Npr⁻ mutants (Figure 8B, lanes 5 and 6, respectively).

DISCUSSION

We have identified regulatory genes in *M. grisea* that affect pathogenicity and aspects of nitrogen metabolism. We postulated the existence of these regulatory genes based on the complex regulation observed for the *MPG1* gene (Talbot et al.,

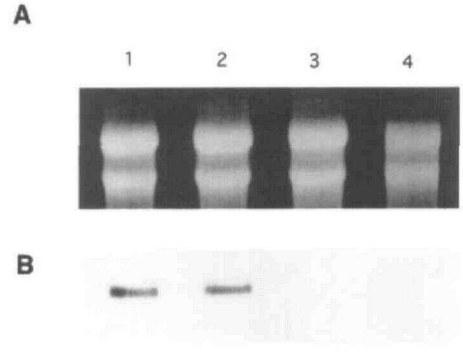

Figure 7. *NPR1* and *NPR2* but Not *NUT1* Are Required for the Full Expression of the *MPG1* Gene during Carbon Starvation.

RNA samples were extracted from carbon-starved cultures, fractionated on a formaldehyde–agarose gel, and transferred to a nylon membrane as described in Methods. Approximately 30 μg of RNA was loaded into each lane. Lane 1 contains wild-type strain 35-R-24; lane 2, Nut1⁻ mutant Guy11nut1-4; lane 3, Npr1⁻ mutant Cl60; lane 4, Npr2⁻ mutant Cl105.

(A) Ethidium bromide–stained gel.

(B) RNA gel blot analysis of the gel shown in **(A)**. The blot was hybridized with the 2.7-kb XbaI-BamHI fragment containing the *MPG1* gene from plasmid pNJT15GXB (Talbot et al., 1993).

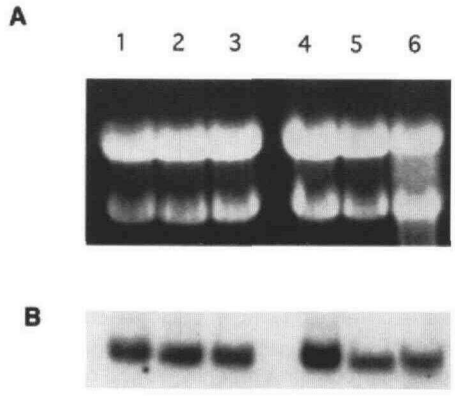

Figure 8. *CPKA* Expression in the Wild Type and Npr$^-$ Mutants during Nitrogen Starvation.

RNA samples were fractionated on a formaldehyde–agarose gel and transferred to a nylon membrane as described in Methods. Approximately 30 μg of RNA was loaded into each lane. Lanes 1 to 3 contain RNA samples extracted from cultures grown in complete medium. Lanes 4 to 6 contain RNA samples extracted from cultures grown in nitrogen-starved medium. Lanes 1 and 4, wild-type strain 35-R-24; lanes 2 and 5, Npr1$^-$ mutant CI60; lanes 3 and 6, Npr2$^-$ mutant CI105.
(A) Ethidium bromide–stained gel.
(B) RNA gel blot analysis of the gel shown in **(A)**. The blot was hybridized with a *CPKA*-specific fragment.

1993; J. Beckerman and D.J. Ebbole, submitted manuscript). A combinatorial genetic screen identified single gene mutations that blocked pathogenicity, prevented growth on a wide range of nitrogen sources, prevented expression of the pathogenicity gene *MPG1*, and abolished up-regulation of the nitrogen-regulated gene nitrate reductase (*NIAD*). The mutations defined two new positive-acting genetic loci that are unlinked from the major transcriptional regulator of NR. *NPR1* and *NPR2* appear to represent novel *trans*-acting regulatory mutations affecting fungal pathogenicity. Interestingly, they may also represent novel genes involved in fungal nitrogen regulation.

Most Genes in Nitrogen Metabolism Are Dispensable for Pathogenicity

In *M. grisea*, we sought regulatory mutations in nitrogen metabolism because the pathogenicity gene *MPG1* was shown to be up-regulated by nitrogen limitation (Talbot et al., 1993). We isolated mutants defective in the utilization of various nitrogen sources by selecting for chlorate-resistant mutants. The recovered mutations include strains with Cnx$^-$, CrnA$^-$, NiaD$^-$, Nir$^-$/NiiA$^-$, and Nut$^-$ phenotypes (Table 1). The distribution of mutant classes appears to be similar to that of *A. nidulans* (Cove, 1976), with the proportion of each class of mutants reflecting the number of the potential target genes. For example, 250 of 380 of our chlorate-resistant *M. grisea* mutants are Cnx$^-$, presumably reflecting a large number of target genes. In *A. nidulans*, there are eight genes required for molybdenum cofactor synthesis. Thirty mutants were of the NirA$^-$/NiiA$^-$ class, which is approximately twice as many as were in the NiaD$^-$ class (Table 1). Finally, phenotypic classes that may represent regulatory genes were represented by fewer mutants (Nr$^-$ and Npr$^-$). Surprisingly, representative chlorate-resistant mutants from each class were fully pathogenic (except for the two Npr$^-$ mutants).

This result suggests that barley plants may contain an abundance of glutamine or that a different nitrogen regulatory pathway is required for fungal growth in the plant. Although our results were performed in barley pathogens, they are completely consistent with the recent finding that a null mutation in the major transcriptional regulator of nitrogen catabolite derepressed genes in *M. grisea*, *NUT1*, is dispensable for pathogenicity on rice (Froeliger and Carpenter, 1996).

NPR1 and *NPR2* Are Regulatory Loci

We identified two novel genes, designated *NPR1* and *NPR2*, that appear to play regulatory roles in pathogenicity and NR. Several lines of evidence support our finding that *NPR1* and *NPR2* appear to be unrelated to the only known wide-domain regulators of NR in filamentous fungi. First, *npr1-1* and *npr2-1* mutations are unlinked from *NUT1*. Second, Npr$^-$ mutants are defective in pathogenicity, whereas other Nr$^-$ mutants (e.g., Nut1$^-$) are fully pathogenic. Third, the *nit-2* gene of *N. crassa* was able to complement a Nr$^-$ strain but not the Npr$^-$ strains. Fourth, in contrast to *NUT1*, *NPR1* and *NPR2* are required for basal levels of *MPG1* transcript accumulation. Preliminary evidence described here suggests that *NPR1* and *NPR2* act at the level of transcript abundance. A cosmid clone that complements *NPR2* has been obtained recently (G. Lau and J.E. Hamer, unpublished data), and molecular cloning of *NPR1* is under way. Regardless of their specific function, the formal genetic analysis presented here suggests these loci act as positive regulatory genes.

NPR1 and *NPR2* must affect a large number of genes. Although only *NPR2* appears to affect nitrate reductase transcript accumulation during induction, mutations in either gene cause a wide range of defects in nitrogen source utilization. A paucity of cloned genes from *M. grisea* prevents us from testing additional nitrogen-regulated genes at this time; however, the observation that the growth phenotypes of Npr$^-$ mutants mimic Nr$^-$ mutants is consistent with a wide-domain regulatory role. Like nitrogen metabolism, pathogenic growth also involves a large number of genes (Valent et al., 1991). We found that Npr1$^-$ and Npr2$^-$ mutants are almost completely defective in pathogenicity. Npr2$^-$ mutants are defective in appressorium formation (G. Lau and J.E. Hamer, unpublished results). We are currently characterizing these phenotypes in more detail; however, the dramatic loss of pathogenicity in these mutants contrasts with the more moderately reduced pathogenicity phenotype of *MPG1* deletion mutants (Talbot et al., 1993). This

finding strongly suggests that the loss of pathogenicity due to *npr1* and *npr2* mutations occurs because a number of pathogenicity genes failed to be expressed. Differential screening or pseudoreversion analysis may be a useful strategy for identifying these genes.

MPG1 Is Subject to Complex Regulatory Controls

The results of our RNA gel blot analysis support the notion that *MPG1* regulation is complex. We found that *NUT1*, *NPR1*, and *NPR2* are involved in *MPG1* expression. Recently, appressorium formation and *MPG1* expression were shown to be inhibited dramatically by growth with 2% yeast extract (J. Beckerman and D.J. Ebbole, submitted manuscript). These results suggest that a nutritionally linked regulatory mechanism may be involved in the expression of *MPG1* and possibly additional genes critical for appressorium formation and pathogenesis. In support of this hypothesis, we found that *NPR1* and *NPR2* also are necessary for *MPG1* expression during carbon starvation. Interestingly, Npr$^-$ strains were not defective in utilizing various sugars (G. Lau and J.E. Hamer, unpublished results) but showed slower growth on oatmeal agar, suggesting that the effects of *NPR1* and *NPR2* on carbon metabolism may be more limiting. Recently, a homolog of the yeast glucose regulatory gene *GRR1* was identified in *M. grisea* from an insertional mutagenesis screen (J. Sweigard, personal communication). The insertion mutant fails to up-regulate *MPG1* (J. Beckerman and D.J. Ebbole, personal communication) but showed few defects in sugar utilization. These results further support the idea of links between metabolic gene regulation and pathogenicity and suggest that a wide variety of mutational approaches can be taken to identify other regulators of pathogenicity.

Nutritional deprivation of various sorts appears to play a role in other fungal morphogenetic switches. For example, invasive growth of *S. cerevisiae* and the formation of pseudohyphae require a change in bud growth and are triggered, in part, by starvation for nitrogen (Gimeno et al., 1992). Similarly, submerged conidiophore formation in *A. nidulans* can be triggered by carbon or nitrogen deprivation (Skromne et al., 1995). In other phytopathogens, the production of toxins (reviewed in Garraway and Evans, 1984) and the expression of virulence determinants also require various types of nutrient deprivation (Rahme et al., 1992; Schulte and Bonas, 1992; Wei et al., 1992; Talbot et al., 1993). Our current hypothesis is that *NPR1* and *NPR2* are part of a nutrition-linked regulatory mechanism for pathogenesis in *M. grisea*.

A Model for Nutrition-Linked Pathogenicity Gene Expression

The relationship we have uncovered among *NPR1*, *NPR2*, *NUT1*, nitrogen metabolism, and pathogenicity is diagrammed in Figure 9. The positive action of *NPR1* and *NPR2* would appear to be epistatic to the action of *NUT1*. This is based on several findings. First, Npr$^-$ mutants show a more pleiotropic phenotype than Nut1$^-$ mutants show because they are defective in both the utilization of exogenous nitrogen sources and in pathogenicity. Second, in contrast to Nut1$^-$ mutants, Npr$^-$ mutants are defective in both the basal level expression of *MPG1* and the induction of *MPG1* during nitrogen limitation. Finally, we recently tested the pathogenicity of Npr$^-$ and Nut1$^-$ double mutants and found that they were nonpathogenic on barley (data not shown). The results support *NPR1* and *NPR2* being epistatic to *NUT1*. However, we cannot rule out the possibility of failure of pathogenicity due to the nonisogenicity of the strains used.

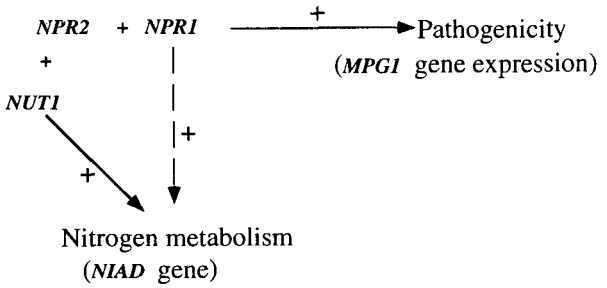

Figure 9. Proposed Genetic Model for Interactions among *NPR1*, *NPR2*, and *NUT1*.

NPR1 and *NPR2* are proposed as *trans*-acting positive regulators of both nitrogen metabolism and pathogenicity. Both genes appear to be necessary for growth on diverse nitrogen sources, and both genes appear necessary for pathogenesis. *NUT1* is proposed to be positively regulating for nitrogen metabolism and is considered dispensable for pathogenicity.

The *NPR* genes may be coregulators of nitrogen metabolism genes or may have a more direct effect on *NUT1*. Interestingly, positive-acting genes with wide-domain regulatory effects other than *areA* and *NIT2* have yet to be found in *N. crassa* or *A. nidulans*. Thus, it will be of interest to test the role of NPR homologs in saprophytic fungi. Finally, we are using insertional mutagenesis and differential screens to identify additional pathogenicity genes in *M. grisea*. It will be of interest to test whether these genes also are regulated by *NPR1* and *NPR2*.

METHODS

Strains, Media, and Genetic Crosses

Strains of *Magnaporthe grisea* used in this study are listed in Table 4. The pedigree of parental strains 35-R-24 and 35-R-56 is shown in Figure 1A. Strain numbering, growth, storage, genetic crosses, and media for *M. grisea* are as described by Crawford et al. (1986). Tanaka minimal medium is as given in Tanaka (1965). Crosses were performed on oatmeal media as described by Crawford et al. (1986). Growth con-

Table 4. *M. grisea* Strains Used in This Study

Strains	Phenotypes	Source
35-R-24	Barley pathogen	Laboratory strain; this study
35-R-56	Barley pathogen	Laboratory strain; this study
Cl60 (*npr1-1*)	ClO_3^r; Nr^-, Pth^-	Spontaneous mutant of 35-R-24; this study
Cl105 (*npr2-1*)	ClO_3^r; Nr^-, Pth^-	Spontaneous mutant of 35-R-24; this study
Guy11nut1-4	Hyg^r, $Nut1^-$	Froeliger and Carpenter (1996)
Guy11397Cl13	Nr^-	M.H. Lebrun, University of Paris–Sud; this study

ditions for nitrogen and carbon starvation were as described by Talbot et al. (1993), with 2% yeast extract being added to complete media to maintain repression of *MPG1* (J. Backerman and D.J. Ebbole, submitted manuscript).

Isolation of the Spontaneous Chlorate-Resistant Mutants

Fertile strains of *M. grisea* that are highly pathogenic on barley were developed (see Figure 1A). Chlorate-resistant mutants were isolated as described previously (Cove, 1976). Conidia from two strains of *M. grisea*, 35-R-24 (*MAT1-2*) and 35-R-56 (*MAT1-1*), were plated onto Tanaka minimal medium supplemented with 5% sodium chlorate and 6 mM urea at a concentration of $\sim 10^5$ conidia per plate. Resistant colonies were recovered 2 weeks after plating, and each mutant was clonally purified by single conidium isolation. Mutants were classified based on growth tests performed on solid Tanaka minimal media supplemented with 6 mM various nitrogen sources. Nomenclature for mutant phenotypes is based on those used for *Aspergillus nidulans*, except for *NUT1* (designated by E. Froeliger, University of Vermont, Burlington, VT), *NPR1*, and *NPR2* (this study).

Infection Assays, Nucleic Acid Manipulation, and Fungal Transformation

Infection assays were performed as described for rice (Talbot et al., 1993), except that 14-day-old seedlings (3 to 4 leaf stage) of barley cultivar Golden Promise were used. Fifteen seedlings were inoculated with each strain tested, and the infections were repeated three times. RNA extractions, RNA gel blots, and *M. grisea* transformations were performed as described previously (Talbot et al., 1993). The *M. grisea NIAD* gene was kindly provided by J. Sweigard (Du Pont Co., Wilmington, DE). Probes for the *CPKA* gene (GenBank accession number U12335) were obtained by polymerase chain reaction, using gene-specific primers.

ACKNOWLEDGMENTS

We are grateful to Drs. Claudio Scazzocchio, Marc-Henri Lebrun (University of Paris), Herb Arst and David Holden (Royal Postgraduate Medical School), Mark Caddick (University of Liverpool), and Nick Talbot (University of Exeter) for useful suggestions and ideas on fungal nitrogen metabolism and pathogenicity. We are also indebted to Dr. Matt Henry (Dow Elanco Inc.) for suggesting the use of barley cultivar Golden Promise. We are especially grateful to Drs. Dan Ebbole (Texas A & M University), Eunice Froeliger (University of Vermont), and Jim Sweigard (Du Pont Co.) for openly communicating and sharing unpublished data. Finally, we thank members of the Hamer laboratory for lively discussions and sharing ideas. This work was supported by grants from the National Science Foundation and a Presidential Faculty Fellow Award to J.E.H.

Received November 17, 1995; accepted February 22, 1996.

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