Identification and Characterization of MPG1, a Gene Involved in Pathogenicity from the Rice Blast Fungus *Magnaporthe grisea*

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Differential cDNA cloning was used to identify genes expressed during infectious growth of the fungal pathogen *Magnaporthe grisea* in its host, the rice plant. We characterized one of these genes, MPG1, in detail. Using a novel assay to determine the proportion of fungal biomass present in the plant, we determined that the MPG1 transcript was 60-fold more abundant during growth in the plant than in culture. Mpg1 mutants have a reduced ability to cause disease symptoms that appears to result from an impaired ability to undergo appressorium formation. MPG1 mRNA was highly abundant very early in plant infection concomitant with appressorium formation and was also abundant at the time of symptom development. The MPG1 mRNA was also expressed during conidiation and in mycelial cultures starved for nitrogen or carbon. MPG1 potentially encodes a small, secreted, cysteine-rich, moderately hydrophobic protein with the characteristics of a fungal hydrophobin. Consistent with the role of the MPG1 gene product as a hydrophobin, Mpg1 mutants show an “easily wettable” phenotype. Our results suggest that hydrophobins may have a role in the elaboration of infective structures by fungi and may fulfill other functions in fungal phytopathogenesis.

INTRODUCTION

The ability of a plant pathogenic fungus to infect and cause disease on its host plant is an intricate process encompassing the entire life cycle of the pathogen. Pathogenicity is therefore a complex phenotype involving such disparate abilities of the fungus as its infection efficiency, the extent to which it colonizes its host, its derangement of plant metabolism, and its capacity for disseminating infective propagules to new host plants.

The complexity of the pathogenic lifestyle is exemplified by the ascomycete fungus *Magnaporthe grisea*. The fungus is a pathogen of a large number of grass species but is best known as the causal agent of rice blast disease, the most serious disease of cultivated rice (Ou, 1985). The infective cycle of the rice blast fungus starts when an asexual spore (a conidium) lands on the leaf surface. The events that follow depend on the prevailing phyllosphere environment and the ability of the spore to enter a complex morphogenetic sequence. Hydration of the conidium results in the release of an adhesive from a periplasmic space at the spore apex which binds it tightly to the hydrophobic leaf surface (Ham et al., 1988). The spore germinates within 2 hr of binding to the leaf surface and forms a short germ tube which differentiates at the tip to produce the infective cellular structure, the appressorium (Howard et al., 1991a). The formation of this cell involves the cessation of apical growth and extensive melanization of the cell wall (Howard and Ferrari, 1989).

Infection proceeds by tight adhesion of the appressorium to the leaf surface, followed by the build-up of a high internal turgor pressure (Howard et al., 1991b) which allows a penetration peg to break through the cuticle and into the underlying epidermal cell layer. This process may involve actin-associated events as actin has been immunolocalized to penetration pegs of *M. grisea* (Bourett and Howard, 1992). Infectious hyphae ramify throughout the underlying epidermal cells differentiating into bulbous, branched secondary hyphae which spread both intercellularly and intracellularly (Heath et al., 1990a, 1990b). At 96 hr after the initial infection, the first visible symptoms become apparent. Small ellipsoid lesions appear on leaves resulting from rapid chlorosis and necrosis of host tissues (Peng and Shishiyama, 1988; Valent et al., 1991). In heavy infection the lesions ultimately coalesce, covering much of the leaf surface. The fungus then sends out aerial conidiophores which sporulate extensively to further spread disease.

The successful colonization of the rice plant by *M. grisea* thus requires a large variety of morphogenetic and metabolic processes. Genetic characterization of pathogenesis has
proceeded by the identification of single gene mutations that bring about a loss of pathogenicity primarily by altering the function or formation of appressoria (for review, see Valent and Chumley, 1991). Conversely, molecular genetic approaches have shown that some putative pathogenicity genes such as cutinase may be dispensable for full pathogenicity (Sweigard et al., 1992a, 1992b). Physiological studies of rice blast disease have identified a number of phytotoxins produced by M. grisea including tenuazonic acid (3-acetyl 5-sec-butyl pyrrolidine-2,4-dione) and pyricularin (for review, see Ou, 1985), although their precise role in pathogenesis awaits further characterization (Lebrun et al., 1990). Thus, many diverse physiological and morphogenetic processes may be classifiable as being mediated by "pathogenicity" genes. The genetic evidence for the complexity of pathogenesis is apparent from the study of Valent et al. (1991) in which polygenic factors governing lesion density and size were observed in progeny segregating for major host or cultivar specificity (avirulence) genes.

Given the complexity of infectious growth, we reasoned that one strategy to identify genes playing important roles in pathogenicity would be to study genes expressed preferentially during growth of M. grisea in the plant. This paper describes the results of a differential cDNA screening designed to identify such genes. One gene, designated MPG1, encodes an mRNA that is ~60-fold more abundant during infectious growth than in conditions used to grow M. grisea in culture. MPG1 is a single copy gene, maps to a new locus on the end of linkage group H (Romao and Hamer, 1992), and is conserved in most if not all isolates of M. grisea. Null mutations at the MPG1 locus produce a reduced pathogenicity phenotype on compatible rice cultivars. The reduced pathogenicity phenotype is associated with the reduced frequency of appressorium development by Mpg1 mutants. DNA sequencing showed that the putative MPG1 gene product is related to the fungal hydrophobins, a recently described group of secreted proteins (Stringer et al., 1991; Wessels et al., 1991a; Lauter et al., 1992; St. Leger et al., 1992). Hydrophobin proteins have been implicated in various aspects of fungal morphogenesis, including the production of aerial hyphae and reproductive structures. Our results suggest these proteins may also play important roles in pathogenesis.

**RESULTS**

**Identification of Abundant mRNAs during M. grisea Infections**

Rice plants of the cultivar CO-39 were infected with the compatible M. grisea isolate Guy-11. The infection was allowed to proceed for 72 hr. At this point few visible disease symptoms were apparent, but considerable host colonization had already occurred. Total RNA was extracted from the infected rice leaves, enriched for poly(A)+ RNA, and used to construct a directional cDNA library (see Methods). A low-density differential screen was then carried out. Replicate filters were hybridized with cDNA probes derived from uninfected rice leaf poly(A)+ RNA and M. grisea-infected rice leaf poly(A)+ RNA. We selected 42 cDNAs that hybridized preferentially to the infection cDNA probe.

These cDNAs were converted to plasmid clones and classified as being of rice or M. grisea origin by DNA gel blot analysis (data not shown). The cDNAs were then classified by their hybridization patterns to RNA gel blots, and representative clones are shown in Figure 1. Blots were prepared with

![Figure 1](image-url)
equal amounts of poly(A)+ RNA from _M. grisea_ grown in axenic culture, uninfected rice leaves, and _M. grisea_-infected rice leaves. NJT-15 (Figure 1A) represents a _M. grisea_ mRNA species expressed abundantly during plant infection. A longer exposure of the autoradiogram is given in Figure 1B, showing that low level expression of the NJT-15 gene can be detected in axenic culture. NJT-23 (Figure 1C) represents an abundant _M. grisea_ mRNA species produced constitutively in culture and during growth in the plant. NJT-70 (Figure 1D) represents a less abundant _M. grisea_ mRNA species expressed at a slightly higher level in the plant than in culture based on estimation of fungal mRNA levels in the plant (see below). NJT-49 (Figure 1E) represents a rice mRNA species produced abundantly during _M. grisea_ infection. NJT-14 (Figure 1F) represents a rice mRNA species found specifically during _M. grisea_ infection.

**Estimation of mRNA Levels in the Plant**

Quantitative measurements of fungal gene expression during growth in the plant is difficult because it is dependent on the relative proportions of fungal and plant biomass. This proportion changes with time and is likely to vary from experiment to experiment. Correlating RNA gel blot signals of fungal genes to known single copy genes was unsuccessful because the level of expression of several of these genes (e.g., _ILV1_ and β-tubulin) was almost undetectable in mRNA preparation from infected plants and too little is known about infectious fungal growth to assume that any gene will be constitutively expressed.

Assuming that the proportion of fungal and plant biomass present at any given time during an infection is equivalent to the proportion of plant and fungal DNA, DNA gel blot analysis can be used to estimate the extent of fungal growth in the plant. We found that plant and fungal DNA could be quantitatively extracted by a single protocol and that DNA extraction by this method was mass dependent (see Methods). Figure 2 shows hybridization of the _ILV1_ probe, a single copy _M. grisea_ gene encoding isoleucine valine synthase (Valent and Chumley, 1991), to total genomic DNA extracted from rice plants 72 hr after inoculation with _M. grisea_. This is the time at which the infection mRNA was routinely extracted and from which the cDNA library was constructed. The hybridization signals from this analysis were compared to a dilution series of DNA samples extracted from _M. grisea_ nuclei (Figure 2). Laser densitometry of the hybridization signals showed that 500 ng of _M. grisea_ genomic DNA gave the same hybridization signal as 5 µg of blast-infected rice genomic DNA. Therefore, 10% of the infected rice DNA is composed of _M. grisea_ genomic DNA. Because DNA extraction is mass dependent, 10% of the biomass of infected leaves can be accounted for as representing _M. grisea_ mycelium. When this value was used to normalize the densitometry data from the RNA gel blot hybridization experiments, the mRNA detected with the NJT-15 clone (Figures 1A and 1B) was 62 times more abundant in the plant than in culture, and the mRNA detected with the NJT-70 clone (Figure 1D) was 3.5 times more abundant.

The biomass estimates were also used to follow the rate of fungal ingress, as shown in Figure 2B. _M. grisea_ biomass appears to have more than doubled between 72 and 96 hr after inoculation. This dramatic increase is consistent with microscopic observations that show very rapid and extensive colonization of the plant leaf area prior to symptom development, which occurs approximately 96 hr after initial infection (Peng and Shishiyama, 1988; Heath et al., 1990a).

We also compared the signals of infection-induced _M. grisea_ cDNAs with NJT-23, a putative constitutively expressed _M. grisea_ cDNA (Figure 1C). Later we show that the NJT-23 transcript is probably not constitutively expressed at all times during...
the infection but is abundant at the 72-hr time point examined in this experiment. Normalization of the hybridization signals suggested that the NJT-15 cDNA represents an mRNA species 40-fold more abundant in the plant than in culture and the NJT-70 cDNA, an mRNA species three times more abundant in the plant than in culture. Thus, the estimates of fungal mRNA levels in the plant normalized relative to another gene are similar to those derived from estimations of fungal biomass.

Characterization and Genetic Mapping of MPG1

The NJT-15 cDNA was selected for detailed characterization. DNA gel blot analysis demonstrated that NJT-15 defined a single copy gene in all host-limited forms and in all pathotypes (physiological races) of M. grisea rice pathogens examined (data not shown). The NJT-15 cDNA clone was used as a hybridization probe for restriction fragment length polymorphism (RFLP) mapping of the gene in the mapping strains 4375-R-26 and 4136-4-3 (Romao and Hamer, 1992), and the results are shown in Figure 3. Segregation of a Sall RFLP detected with NJT-15 was followed relative to RFLPs detected around MGR586 dispersed repeated sequences which have been genetically mapped in the M. grisea genome (Hamner and Givan, 1990; Romao and Hamer, 1992). The RFLP mapped to a new site near the end of linkage group H (designation of Romao and Hamer, 1992). The genetic mapping was confirmed by hybridization of NJT-15 to a single chromosomal-sized DNA band in a pulsed field gel separation of M. grisea chromosomal-sized DNAs (Figure 3B) previously determined to be linkage group H (N. J. Talbot and J. Romao, unpublished data; Romao and Hamer, 1992). This new genetic locus was designated MPG1.

Inactivation of MPG1 Leads to a Reduction in Pathogenicity on Rice

The MPG1 gene was inactivated by a one-step gene replacement strategy (Rothstein, 1983), as shown in Figure 4. A full-length genomic clone of the MPG1 locus was obtained from a genomic library of strain Guy-11, restriction mapped, and the orientation of the cDNA clones determined (Figure 4A). To construct the gene replacement vector pNT400, 3.5-kb flanking regions, 5' and 3' of the MPG1 coding region, were individually subcloned. The flanks were ligated together, and a selectable marker gene, a hygromycin resistance gene (Hph), was inserted between them (see Methods). The entire construct was assembled in pBluescript KS+ (Stratagene) and linearized with NotI prior to transformation into strain Guy-11. Transformation of this construct allows homologous integration of the vector at the MPG1 locus, as illustrated in Figure 4A, leading to direct replacement of the MPG1 coding region with the Hph gene.

Sixty hygromycin-resistant Guy-11 transformants were selected and analyzed by DNA gel blot hybridization using pNJT-15. Two transformants TM400-2 and TM400-5 showed the predicted mpg1::Hph gene replacement, failing to hybridize to the pNJT-15 cDNA insert as shown in Figure 4C. The presence of the Hph gene in these transformants was confirmed by DNA gel blot analysis (Figure 4B). The production of viable mpg1::Hph transformants showed that the MPG1 gene is nonessential for growth of M. grisea in culture.

Pathogenicity assays were carried out using the wild-type strain Guy-11, the two independently obtained mpg1::Hph transformants (TM400-2 and TM400-5), and two independently obtained transformants in which the gene replacement vector had integrated into the genome at an ectopic site (TM400-1 and TM400-10). The results are shown in Figure 5. The wild-type strain Guy-11 caused large spreading lesions on rice leaves (Figure 5A) as did the transformants TM400-1 and TM400-10 (Figures 5D and 5E). In contrast, the Mpgl null mutants TM400-2 and TM400-5 caused very reduced symptoms with only a few lesions visible (Figures 5B and 5C). A more quantitative analysis of pathogenicity was carried out by measuring lesion densities on infected rice leaf tips (see Methods).
Figure 4. Inactivation of the MPG1 Gene by a One-Step Gene Replacement Strategy.

(A) Restriction map of the MPG1 locus with the orientation and position of the NJT-15 cDNA. A genomic clone of this region, λ15G-1, was isolated, and 3.5-kb flanking restriction fragments were subcloned from 5' and 3' of the coding region. A selectable marker gene, HPH, conferring hygromycin resistance, was inserted between the flanks to produce pNJT400. This construct was transformed as a linear restriction fragment into the wild-type M. grisea strain Guy-11. The double crossover event results in the inactivation of the MPG1 gene by complete replacement of the coding region with the HPH gene. B, BamHI; C, SacI; E, EcoRI; H, HindIII; M, Smal; P, PstI; S, SalI; X, XhoI; Xb, XbaI.

(B) and (C) DNA gel blot analysis of hygromycin-resistant transformants containing the desired integration event. DNA was extracted, digested with XbaI, fractionated on 0.8% agarose gels, blotted, and hybridized with a 2.6-kb XbaI fragment excised from pNT25 containing the HPH gene cassette (B) and the 765-bp cDNA insert from the MPG1 cDNA clone pNJT15 (C). Two transformants, TM400-2 and TM400-5, had undergone the desired deletion mutation at MPG1, shown by lack of hybridization of the pNJT15 insert to the expected 8.4-kb XbaI genomic restriction fragment. Confirmation of transformation was shown by hybridization of the HPH gene to a 2.6-kb genomic restriction fragment. The wild-type recipient strain Guy-11 and a transformant in which pNJT-400 had integrated at an ectopic site in the genome (TM400-10) are also shown.
Conidial suspensions were prepared from Mpg1 deletion mutants and control strains and sprayed onto 14-day-old seedlings of the compatible rice cultivar CO-39. The plants were incubated to allow disease development for 96 hr. Leaves shown were from plants inoculated with the following strains.

(A) Wild-type Guy-11.
(B) Mpg1 strain TM400-2.
(C) Mpg1 strain TM400-5.
(D) Ectopic transformant TM400-1.
(E) Ectopic transformant TM400-10.

Mpg1 mutants produced significantly fewer lesions. The experiment was repeated four times with identical results using 160 seedlings per experiment.

Mean lesion density was reduced from 37.4 per 5-cm leaf tip in the wild-type isolate Guy-11 to 7.5 per 5-cm leaf tip in TM400-2 ($t = 12.65, P < 0.001, df = 78$). Mean lesion density was similarly reduced in TM400-5 to 7.3 per 5-cm leaf tip, but was unchanged in both TM400-1 and TM400-10.

**mpg1::Hph Strains Show a Reduced Ability To Form Appressoria**

The reduced lesion density suggested that the Mpg1 null mutants were defective in early steps in the infection process. The ability of the mpg1::Hph strains to form appressoria was assayed on Teflon (DuPont) membranes (Harmer et al., 1988). Figure 6A shows differentiated appressoria from the wild-type strain Guy-11 in which 70.8% of the conidia examined ($n = 1200$) produced appressoria. The pyriformed three-celled conidia germinate and form a round melanized appressoria. The transformants TM400-1 and TM400-10 were unchanged in their ability to form appressoria relative to strain Guy-11 (data not shown). Figure 6B shows the reduced frequency of appressorial formation in strain TM400-2. Conidia from this strain germinate normally (data not shown) but failed to form appressoria at a high frequency. We found that only 18.3% of conidia examined on Teflon ($n = 1200$) from TM400-2 formed appressoria ($\chi^2 = 39.09, P < 0.001$). Similarly, 20.6% of conidia ($n = 1200$) from TM400-5 produced appressoria ($\chi^2 = 35.96, P < 0.001$). The inset (Figure 6C) shows a close-up of a typical conidium that failed to produce an appressorium. A long germ tube has formed that appears to have undergone multiple hooking and swelling events. Previous studies (Bourett and Howard, 1990) have shown that hooking and swelling of the germ tube occur prior to appressorium formation. Thus, Mpg1 null mutants appear to undergo primary differentiation events that
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Time after inoculation (h)
12 18 24 48 72 96

Figure 7. Temporal Expression of MPG1 during Rice Blast Pathogenesis.

Total RNA (5 μg per lane) was extracted from leaves of blast-infected rice plants at six time points following their inoculation; 12, 18, 24, 48, 72 and 96 hr (h). The RNA was fractionated by gel electrophoresis under denaturing conditions, blotted to Hybond N membranes, and hybridized with the following.

(A) pNJT-15 (MPG1).
(B) A M. grisea cDNA pNJT-23 found abundantly during growth in the plant.
(C) λrm8, a genomic clone containing the ribosomal DNA repeat unit from M. grisea, which hybridizes to both rice and fungal rRNA.

MPG1 mRNA is abundant early in the infection process.

Temporal Expression of MPG1 during M. grisea Pathogenesis

The MPG1 gene was identified by its high expression during growth in the plant by M. grisea. Paradoxically, the phenotype of the Mpg1 mutants produced by gene deletion/replacement was a defect in appressorial formation, an early event in pathogenesis. We decided to examine the relative mRNA abundance of the MPG1 gene at various times during rice blast infection, ranging from the onset of infection (12 hr after plant inoculation) to full symptom expression (96 hr after inoculation). Results from RNA gel blot analysis using the MPG1 gene (pNJT-15) as a hybridization probe are shown in Figure 7. Surprisingly, the MPG1 message could be detected as early as 12 hr following inoculation of rice plants with M. grisea but was not detected at 18, 24, or 48 hr postinoculation. However, the MPG1 transcript was again detected 72 to 96 hr postinoculation, concomitant with the first signs of symptom expression. Prior to a point 48 hr postinoculation, no fungal biomass could be detected in infected rice leaves (Figure 2B). Thus, at the far earlier time point of 12 hr after inoculation, only a minute amount of fungal biomass is probably present in and on infected rice leaves. This agrees with microscopic analysis of blast infections which show that the fungus has elaborated an appressorium and is just beginning primary plant infection 12 hr after plant inoculation (Heath et al., 1990a). The detection of the MPG1 transcript in total RNA at the 12-hr time point suggests that the MPG1 gene is very highly expressed during the early stages of plant colonization. Control hybridization were performed with the highly expressed cDNA clone NJT-23 (Figure 1C) and with the ILV1 and TUB1 genes (encoding isoleucine valine synthase and β-tubulin, respectively; data not shown). Transcripts of these genes were undetectable until 72 hr after inoculation. The NJT-23 transcript was most abundant 72 hr after initial plant infection (Figure 7B), a time concomitant with the presence of extensive amounts of fungal biomass in the plant (Figure 2), but was undetectable at earlier or later times during disease development.
Figure 9. Transcriptional Organization and Sequence of the MPG1 Locus.

(A) Restriction map of the MPG1 locus showing orientation of the cDNA clone pNJ7-15 and the genomic subclone pNJTG15XB. B, BamHI; E, EcoRI; H, HindIII; M, Smal; P, PstI; S, SalI; V, EcoRV; X, Xhol; Xb, Xbal.

(B) DNA sequence of the transcriptional unit of MPG1. The transcription initiation site was determined by primer extension mapping. The two major 5' ends are indicated by the arrow at positions +1 and +4. A putative TATA box (~52) in the 5' region of the gene is overlined. A 77-bp intron was identified by comparison of the cDNA and genomic sequence and is shown in lowercase letters. The hexanucleotide polyadenylation signal sequence in the 3' untranslated region of the gene is underlined at position 835. The poly(A) addition sites are underlined and were deduced from the cDNA sequences of two independent clones.
were aligned based on the conserved cysteine residues (shown in bold) using the GCG PILEUP program (Devereaux et al., 1984). Highly conserved amino acid residues are shown in uppercase letters and as a consensus sequence.

Figure 10. Comparison of the Polypeptide Sequence Predicted by MPG1 with the Fungal Hydrophobins.

The predicted MPG1 polypeptide sequence was aligned with the known hydrophobins. Sc1, Sc3, and Sc4 are proteins from S. commune (Wessels, 1992); segA, rodA, and Eas are translated from genes of M. anisopliae (St. Leger et al., 1992), A. nidulans, and N. crassa (Bell-Pederson et al., 1992; Lauter et al., 1992); cerato-ulmin is a peptide from O. ulmi (Bolyard and Sticklen, 1992; Stringer and Timberlake, 1993). The sequences were aligned based on the conserved cysteine residues (shown in bold) using the CCG PILEUP program (Devereaux et al., 1984). Highly conserved amino acid residues are shown in uppercase letters and as a consensus sequence.

**MPG1 Is Expressed during Nutrient Starvation and Conidiation**

We reasoned that the elevated expression of MPG1 during rice blast infection could be due to regulation by either an exogenous plant signal and/or by nutrient deprivation. To distinguish between these possibilities, strain Guy-11 was grown in complete media (CM) for 4 days. The mycelium was then removed with various landmark features of the transcription unit and/or by nutrient deprivation. To distinguish between these possibilities, strain Guy-11 was grown in complete media (CM) for 4 days. The mycelium was then removed (for details, see Methods). RNA was extracted from the mycelium and gel blots were prepared and hybridized with the 

**MPG1 Encodes a Hydrophobin-like Protein**

A detailed map of the MPG1 locus is illustrated in Figure 9A, showing the positions of the NJT-15 cDNA and the pNJT15GX8 genomic subclone. DNA sequence information was obtained from both of these clones, encompassing the entire 2.7-kb region of the MPG1 locus which was deleted in the mpg1::Hph transformants. RNA gel blot analysis with pNJT15GX8 confirmed that only a single 850-bp mRNA was transcribed from this region. The longest open reading frame identified within this 2.7-kb XbaI-BamHI fragment was 336 bp long and predicted a protein of 112 amino acids with a molecular mass of 11.5 kD. The DNA and predicted amino acid sequences together with various landmark features of the transcription unit of MPG1 are shown and described in Figure 9B. We noted that the N terminus of the predicted MPG1 protein contained a tract of amino acids with features consistent with those of a signal sequence (Heijne, 1983), suggesting that MPG1 encodes a secreted peptide. A single 77-bp intron was located in the MPG1 gene by comparison of the genomic and cDNA sequences containing consensus fungal splice sequences (Ballance, 1986).

The MPG1 sequence predicted a small secreted, moderately hydrophobic protein containing eight cysteine residues. These features are characteristic of a class of proteins known as the hydrophobins which have been recently identified in Schizosaccharomyces pombe (for review, see Wessels, 1992), Aspergillus nidulans (Stringer et al., 1991), and Neurospora crassa (Black-Pederson et al., 1992; Lauter et al., 1992). The amino acid sequences of the hydrophobins have two main distinguishing characteristics: the conservation of the spacing of eight cysteine residues and the conservation of hydrophobic domains within the polypeptide sequence. Computer-generated amino acid sequence alignments of MPG1 with the known hydrophobin amino acid sequences, given in Figure 10, showed a similar spacing of the cysteine residues in MPG1. Figure 11 shows an alignment of the hydrophobic plot of the MPG1 amino acid sequence with those of the known hydrophobins. This comparison shows that MPG1 has the characteristic hydrophobic profile of a hydrophobin.
Figure 11. Hydropathy Plots of the Fungal Hydrophobins.

The hydropathy plot of MPG1 calculated according to Kyte and Doolittle (1982) was compared to those of Sc1, Sc4, Sc3, RodA, Eas, ssGA, and cerato-ulmin (C-U). Hydrophobic regions are shown above the x-axis and hydrophilic regions beneath. The C-U hydropathy plot is offset to the right assuming cleavage of the secretory signal sequence from the mature peptide. Hydropathy plots were produced by the program DNA strider 1.0 (Marck, 1988).

However, MPG1 does appear to be the least hydrophobic of the known hydrophobins.

**mpg1::Hph Strains Show an “Easily Wettatable” Phenotype**

Null mutations in rodA or Eas produce an “easily wettatable” phenotype (Stringer et al., 1991; Bell-Pederson et al., 1992). This is very pronounced in the case of Eas mutants where conidia clump together and cultures become severely water-logged. The easily wettatable phenotype in rodA~ mutants leads to a slightly less pronounced water-soaked phenotype. These phenotypes are thought to be caused by a loss of surface hydrophobicity, brought about by the absence of the spore rodlet layer (Stringer et al., 1991; Bell-Pederson et al., 1992).

To check the hydrophobicity of the Mpg1 mutants, we placed 200-μL drops of water on the surface of the wild-type and the two mpg1::Hph M. grisea strains (Figure 12). The water was allowed to stand for 12 hr. After 12 hr, the water drop was still suspended on the hydrophobic surface of the wild-type strain Guy-11 (Figure 12A) but had soaked into the surface of both mpg1::Hph strains (TM400-2 is shown in Figure 12B), leaving a pronounced water-soaked mark. We conclude that the MPG1 gene product contributes to cell surface hydrophobicity of aerial hyphae of M. grisea.

**DISCUSSION**

A low density cDNA screen allowed the identification of a number of cDNA clones representing mRNAs expressed specifically or at an increased level during rice blast disease. The induction of host plant gene expression during pathogenesis is a well studied phenomenon and appears to represent a general stress response to fungal invasion (Bowles, 1990). It is likely that at least some of the plant cDNA clones identified in our screen correspond to some of these well known genes. This cDNA screen also allowed the identification of genes from the fungal pathogen M. grisea. In particular, a gene designated MPG1 was identified in the blast fungus that is highly expressed during infectious growth and appears to be required for appressorium formation and full symptom development. We have presented strong evidence that the probable MPG1 gene product is a small secreted protein with the characteristics of a fungal hydrophobin.

Little is known concerning the physiology of invasive fungal growth in plants. Several researchers have documented that in compatible interactions fungi ramify rapidly throughout the plant leaf area (Heath et al., 1990a, 1990b; Hardham, 1992). We confirmed these observations using a simple and quantitative measure of fungal biomass during growth in the plant. Our analysis demonstrated that 3 days after inoculation up to 10% of the biomass in infected plant leaf tips can be

**Figure 12. Mpg1 Null Mutants Show an “Easily Wettatable” Phenotype.**

Distilled water (200 μL) was placed on the surface of cultures of M. grisea growing on the surface of CM agar, and the plates were incubated for 12 hr at room temperature. A block of agar is pictured next to the site where the drop was placed.

(A) The wild-type strain Guy-11.

(B) The Mpg1 null mutant TM400-2. Bar = 4 mm.

The water droplet remained suspended on the surface of the Guy-11 culture but soaked into the mycelium of the Mpg1 mutant.
attributed to *M. grisea*. Furthermore, this proportion appears to increase dramatically as infection continues. DNA gel blot biomass measurements can be used to quantify race-specific interactions and is broadly applicable to analyzing various properties of infectious growth, including the effects of various antifungal chemicals. The technical simplicity of this approach contrasts with other methods that utilize fungal specific polymers, such as chitin or ergosterol, or rely on the use of transformed fungal pathogens carrying reporter gene constructs.

**The Role of MPG1 in Pathogenesis**

*MPG1* was selected as a gene likely to be important in the establishment of plant infection because of its high level of expression in the plant and its conservation in a broad collection of different races and host-specific forms of *M. grisea*. Gene deletion/replacement mutants of *MPG1* are severely reduced in their ability to cause disease symptoms on susceptible rice cultivars due to an inability to form appressoria. In this report, we have shown that the probable *MPG1* gene product has characteristic features of a fungal hydrophobin. These include conservation of the spacing of eight cysteine residues, including a conserved tripeptide, CCN, present in seven of the eight known hydrophobins, and conservation of hydrophobic domains throughout the polypeptide. *MPG1* also shows a high degree of amino acid similarity, including a large block of identical amino acids, to the *S. commune* hydrophobins and *ssgA* at its C terminus. Furthermore, the *MPG1* null mutants have an "easily wettable" phenotype, suggesting a reduction in cell surface hydrophobicity, similar to that observed in *rodA*− and *Eas*− mutants of *A. nidulans* and *N. crassa*.

The highest levels of expression of *MPG1* during pathogenesis were found to be very early in the infection process during appressorial development (12 hr after plant inoculation) and again very late during the disease cycle at a point coincident with symptom expression. The finding that *MPG1* transcript levels are very abundant during appressorial differentiation and early plant infection is consistent with the observation that * MPG1* mutants are impaired in their ability to undergo appressorial development. Although a generalized role for hydrophobins in the emergence of aerial structures in fungi has been proposed (Chasan, 1991; Wessels, 1992; Wösten et al., 1993), this model would not readily explain the role of these molecules in plant pathogenesis.

Appressorial development occurs in an aqueous environment and is triggered by interactions with a hydrophobic surface (Hamer et al., 1988). Indeed, the mechanical action of appressoria appears to require the influx of water to create the high internal cell turgor pressure needed for penetration (Howard et al., 1991b). According to recent models for hydrophobin function, the *MPG1* hydrophobin would not be expected to accumulate in appressorial cell walls in an aqueous environment. Rather, it would be secreted onto the plant surface and most probably bind to the leaf cuticle through hydrophobic interactions. Recently, it has been shown that purified hydrophobins can self-assemble at any hydrophilic/hydrophobic interface (Wösten et al., 1993). Such an interface may be present between the infecting fungal cell and the plant surface and thus *MPG1* could play a role in attachment, infection court preparation, or topological signaling (for review, see Mendgen and Deising, 1993). Experiments to localize and study the accumulation of the *MPG1* gene product during the infection process are currently underway.

The late expression of *MPG1* during growth in the plant and the recent report that the wilt toxin cerato-ulmin is a fungal hydrophobin (Stringer and Timberlake, 1993) point to another potential role for the *MPG1* protein. Late in pathogenesis the fungus is causing disease symptoms that, in a heavy infection such as the one studied here, include the production of large spreading lesions across the leaf surface and a general desiccation and wilting of the leaf tissue. This phenotype is coincident with high levels of *MPG1* transcript accumulation and could potentially be caused by secretion of large amounts of a small hydrophobic protein that could block xylem function. However, it is difficult to test for the desiccation of leaves by the *Mpg1* null mutants due to their infection deficiency phenotype. We are currently investigating whether the *MPG1* hydrophobin can exert phytopathic effects.

**Regulated Expression of MPG1**

We also showed that *MPG1* transcript levels were elevated during conidiation and that *Mpg1* mutants are impaired in their ability to conidiate, suggesting a role for the gene product in conidiation. Both the *rodA* and *Eas* genes encode hydrophobins that form the rodlet layer of conidial cell walls in *A. nidulans* and *N. crassa*, respectively (Stringer et al., 1991; Lauter et al., 1992), but neither mutant is impaired in conidiation. The *MPG1* gene product may be fulfilling a different role from *rodA* and *Eas* during conidial development. Thus, while the presence of the *MPG1* gene contributes to cell surface hydrophobicity, it may not encode the major rodlet protein. Studies are in progress to localize and characterize the rodlet proteins of *M. grisea*.

Hydrophobin genes are regulated by a diverse array of regulatory genes and environmental cues. In the case of *MPG1*, transcript levels were elevated in RNA from cultures that had been starved for either a carbon or a nitrogen source. This suggests that one of the main environmental cues for appressorial morphogenesis may be nutrient starvation. Consistent with this idea, the *ssgA* gene from the entomopathogen *M. anisopliae* is similar to *MPG1* in that it is regulated by nutrient deprivation and is also highly expressed during appressorium formation (St. Leger et al., 1992). The *S. commune* dikaryon-specific hydrophobins *Sc1* and *Sc4* are regulated by the mating-type genes and by wide domain regulators essential for basidioecarp elaboration (Wessels et al., 1991b; Wessels, 1992). It has been proposed that the *rodA* gene from *A. nidulans* is controlled by the early regulator of conidiation, *BrlA*.
The filter paper was placed mycelium-side down onto complete medium was prepared from some pads immediately. For the remaining pads, observed but the hyphae in the culture were not darkly pigmented. The laboratory of J.E. Hamer (Purdue University, West Lafayette, IN). Standard procedures for the culture and storage of M. grisea were used O.lO/o
Fungal Isolates and Culture Conditions

Strains of Magnaporthe grisea described in this study are stored in the laboratory of J.E. Hamer (Purdue University, West Lafayette, IN). Standard procedures for the culture and storage of M. grisea were used (Crawford et al., 1986) except complete medium (CM) was 10 gl-l glucose, 2 gl-l peptone, 1 gl-l yeast extract, 1 gl-l casamino acids, 0.1% (v/v) trace elements, 0.1% (v/v) vitamin supplement, 6 gl-l NaN03, 0.5 gl-l KCI, 0.5 gl-l MgSO4, 1.5 gl-l KH2P04, pH 6.5.

M. grisea strain Guy-11 was grown in CM for 4 days, at 23°C, in the dark with vigorous aeration. At this time mycelium was removed by filtration, washed three times with sterile distilled water, and transferred to either CM-C containing all the constituents of CM except glucose, yeast extract, peptone, and casamino acids or CM-N containing all the constituents of CM except sodium nitrate, yeast extract, and casamino acids. Mycelium was incubated in this media for 12 hr with vigorous aeration before removal for RNA extraction.

To produce synchronous M. grisea conidiophores, a 2-cm2 agar block of strain Guy-11, grown on oatmeal agar, was fragmented in a blender and used to inoculate 50 mL of CM. After 2 days of growth at room temperature with shaking at 200 rpm, the culture was again blended and used to inoculate three 50-mL CM cultures, and these were grown for 2 days. At this stage some green pigmentation was observed but the hyphae in the culture were not darkly pigmented. Cultures were harvested by vacuum filtration onto 7-cm diameter filter paper circles (Whatman No. 541) to produce a thin mycelial pad. RNA was prepared from some pads immediately. For the remaining pads, the filter paper was placed mycelium-side down onto complete medium agar plates and placed in a 25°C-incubator with constant illumination. After 1 day, hyphae began to protrude through the filter paper. After 3 days, a uniform layer of aerial hyphae was present. After 5 days, a dense layer of gray aerial hyphae was observed with many mature and developing conidiophores present. The filter paper was removed from the agar plate to a hard surface with the aerial hyphae-side up. A razor blade was used to shave the aerial hyphae from the filter paper, and the aerial material was used immediately to prepare RNA.

METHODS

Nucleic Acid Isolations and Analyses

Rice leaves were excised and quickly frozen in liquid N2 for RNA extraction. Leaves were routinely stored in this form at -80°C. RNA was extracted from plant tissues by a guanidine thiocyanate method (Sambrook et al., 1989). RNA was isolated from M. grisea by the method of Timberlake (1960). RNA was poly(A)+ enriched by oligo(dT) cellulose chromatography (Pharmacia) following manufacturer's suggestions. Small scale enrichment of poly(A)+ RNA was performed using the Polyattract kit (Promega). Total or poly(A)+ RNA samples were denatured in 60% formamide, 2.2 M formaldehyde, 1 x Mops/EDTA buffer (1 x Mops/EDTA is 20 mM 3-(N-morpholino)-propanesulfonic acid, 5 mM sodium acetate, 1 mM EDTA) at 65°C for 15 min. Gel electrophoresis was performed on 1.2% agarose gels containing 2.2 M formaldehyde in 1 x Mops/EDTA. Gels were blotted onto Hybond-N (Amersham) and hybridizations were conducted according to manufacturer's suggestions.

Genomic DNA extractions were carried out from rice leaves by the method of Dellaporta et al. (1983) and from M. grisea as described by Talbot et al. (1993).

Mass dependence of DNA extraction from rice and fungal tissues was shown in the following way. Two grams of rice leaf tissue or 2 g of fungal mycelium was taken, ground in liquid NP, and DNA extracted by the method of Dellaporta et al. (1983). A total of 10 replicates was performed. The extracted DNA was quantified in a TKO-100-min fluorimeter (Hoefer Scientific Instruments, San Francisco, CA) using the DNA-specific dye Hoechst 33258. Quantification was confirmed by spectrophotometry and direct visual inspection of ethidium bromide-stained DNA separated on agarose gels. Two grams of rice tissue and 2 g of fungal mycelium both typically yielded 400 μg (plus or minus 50 μg) (data not shown). Total genomic DNA was then extracted from rice plants of cultivar CO-39 that had been inoculated with M. grisea strain Guy-11 at various time points during pathogenesis. The DNA was quantified, digested with restriction enzymes, fractionated by electrophoresis, blotted to nylon membranes, and probed with a single-copy M. grisea gene and then a single-copy rice gene, as described in Figure 2. DNA hybridization probes were labeled by the random primer method (Feinberg and Vogelstein, 1983) using the Stratagene Prime-It kit. DNA gel blot hybridizations were conducted by standard methods (Sambrook et al., 1989) and washed as described previously.
Intact chromosomal-sized DNA was prepared from *M. grisea* protoplasts and separated by contour clamped homogeneous electric field electrophoresis (CHEF) as described by Hamer et al. (1989). Contour clamped homogeneous electric field electrophoresis was carried out for 120 hr at 45 V with a pulse time of 90 min at 4°C. DNA was subsequently transferred to nylon membranes as described above.

Autoradiographs were analyzed by laser densitometry using an Ultrascan II (LKB Instruments, Uppsala, Sweden) at 630 nm. Absorbance values were integrated using the internal integrator function. The data were subsequently analyzed using Gelscan software (LKB-Pharmacia) on an IBM PC-AT.

cDNA and Genomic Library Constructions

The blast-infected rice cDNA library was constructed in λ GEM-4 (Promega) by an adaptation of the methods of Brown and Kafatos (1988) and Polites and Marrotti (1986). Poly(A)⁺ RNA (10 µg) was annealed to an XbaI-oligo(dT) primer adaptor. First strand cDNA synthesis was performed in modified RT buffer (0.8 mM dGTP, dATP, dTTP, 4 mM Na₂HPO₄, 8 mM MgCl₂, 40 mM KCl, 10 mM DTT, 100 mM Tris-HCl, pH 8.7, 50 µg mL⁻¹ actinomycin D) in the presence of 0.8 mM 5-methyl dCTP (Pharmacia) by an adaptation of the methods of Brown and Kafatos (1988). Poly(A)+ RNA (10 µg) was annealed to oligo(dT) primer and reverse transcriptase (Gibco-BRL), for 1 hr at 40°C. DNA:RNA hybrids were collected by ethanol precipitation, and second strand synthesis carried out in a reaction mixture containing the following: 100 mM Hepes, pH 7.6, 6 mM MgCl₂, 10 mM DTT, 60 mM KCl, 0.5 mM dATP, dGTP, dTTP, 0.15 mM β-NAD, 100 µCi α-³²P-dCTP, 150 units DNA polymerase I, 6 units RNaseH, 10 units E. coli DNA ligase, for 1 hr at 14°C. The DNA was phenol extracted, ethanol precipitated, and EcoRI linkers ligated to the double stranded DNA. The cDNA inserts were digested with EcoRI and XbaI and size fractionated prior to ligation to λ GEM 4 and packaging, which were carried out according to manufacturer's suggestions (Promega).

Genomic DNA was isolated from purified nuclei prepared from strain Guy-11 as described by Hamer et al. (1989). The DNA was partially digested with Sau3A1 and cloned into λ GEM11 Xhol half-site arms according to manufacturer's instructions (Promega).

cDNA Library Screening

Low-density screening of a total of 5000 plaque-forming units of the cDNA library was carried out. Duplicate nitrocellulose filters were prepared according to standard methods (Sambrook et al., 1989). Radiolabeled cDNA probes were constructed by an adaptation of the first strand cDNA synthesis protocol described above. One microgram of poly(A)⁺ RNA was annealed to oligo(dT) primer and reverse transcribed in RT buffer in the presence of 50 µl α-³²P-dCTP for 1 hr at 40°C. The probes were purified through Biogel P50 columns (Bio-Rad) constructed in 5-ML disposable pipettes. Plaque lift filters were prehydrized for 4 hr at 65°C in 6 x SSPE (1 x SSPE is 0.15 M NaCl, 10 mM sodium phosphate, 1 mM EDTA, pH 7.4), 2% SDS, 5 x Denhardt's buffer (1 x Denhardt's solution is 0.02% Ficoll, 0.02% PVP, 0.02% BSA). Hybridizations were conducted under the same conditions for 20 hr. The filters were washed under high-stringency conditions (65°C in 0.1% SDS, 0.1% sodium pyrophosphate [PP], 10 mM Na₂HPO₄,1 mM EDTA, pH 7.4) and exposed to x-ray film (Amersham). *Escherichia coli* KW251 (Promega) was routinely used for the propagation of bacteriophage λ.

Genetic Mapping

Restriction fragment length polymorphism (RFLP) mapping was carried out using progeny from the 7-R cross between *M. grisea* strains 4136-4 and 4375-R-26 (Romao and Hamer, 1992). DNA was isolated from the parents and progeny, digested with Sall, and probed with pNJT-15. The segregation of an RFLP detected between the parental isolates was compared with the segregation of RFLPs around MGR586 dispersed repeated elements (Hamer and Givan, 1990; Romao and Hamer, 1992).

Construction of the Gene Replacement Vector pNT400

The gene replacement vector was constructed as outlined in Figure 3. All plasmid clones were constructed in pBluescript SK⁺ (Stratagene). A full-length genomic clone of MPG1, λ 15G-1, was selected by screening the Guy-11 genomic library with pNJT-15. A 5' and 3' proximal 6.25- and 4.5 kb BamHI fragments were subcloned to create pNT108 and pNT211, respectively. XbaI linkers were added to the unique EcoRV site in pNT211 and a 3.4-kb BamHI/XbaI fragment was subcloned and designated pNT220. A 3' 3.4-kb XbaI fragment derived from pNT108 was then cloned into the unique XbaI site in pNT220. The resulting plasmid contained 3.4-kb flanking regions from both 5' and 3' of the MPG1 coding region as a single 68-kb BamHI fragment. This was subcloned into a modified version of pBluescript SK⁺ in which the XbaI site had been removed and designated pNT320. The hygromycin resistance (*Hph*) gene was excised as a 2.6-kb SacI-XbaI fragment from pAN7-1 (Punt et al., 1987). XbaI linkers were added, and the fragment was inserted into the unique XbaI site of pNT320 to give the gene replacement vector pNT400. The insert of this construct is shown in Figure 3B. E. coli strains DH5a (Gibco-BRL) and XLI-Blue (Stratagene) were routinely used for the propagation of plasmids. Recombinant DNA techniques were carried out according to standard protocols (Sambrook et al., 1989).

Fungal Transformations

Transformation of *M. grisea* was carried out by an adaptation of the previously described methods (Parsons et al., 1987; Leung et al., 1990). Briefly, a 2.5 cm² square of *M. grisea* strain Guy-11 mycelium was cut from the surface of an oatmeal agar plate, macerated in 50 mL of CM liquid media, and incubated overnight at 23°C on a rotary shaker at 120 rpm. The culture was blended again and incubated for a second 24-hr period. The culture was harvested by filtration and protoplasts produced by Novozym 234 digestion (Novo Nordisk, Danbury, CT) in OS buffer (1.2 M MgSO₄, 10 mM Na₂HPO₄/NaH₂PO₄, pH 5.8). Protoplasts were collected at the interface of OS overlayed with ST buffer (0.6 M sorbitol, 0.1 M Tris-HCl, pH 7.0) after centrifugation at 1750g. The protoplasts were washed in STC buffer (1.2 M sorbitol, 10 mM Tris-HCl, pH 7.5, 10 mM CaCl₂). Transformation was carried out using 5 x 10⁶ protoplasts, with 2 µg DNA, in the presence of 1 µL PTC buffer (60% polyethylene glycol 4000, 1 M sorbitol, 10 mM Tris-HCl, pH 7.5, 10 mM CaCl₂). The gene replacement vector pNT400 was transformed as a linear NotI fragment. Protoplasts were incubated with PTC for 5 min and then added to 150 mL of molten CM agar, osmotically stabilized by addition of 1 M of sucrose, and poured to 150-mm plates. Protoplasts were allowed to regenerate for 16 hr at 23°C. At this time a selective overlay of 1% CM agar containing 200 µg mL⁻¹ hygromycin B (Boehringer Mannheim) was added, and the plates were incubated...
for 7 days at 30°C in the dark. Monoclonal isolations were conducted on all hygromycin-resistant transformants.

Assays for Infection-Related Morphogenesis

Appressorial formation by M. grisea was observed on Teflon membranes (Du Pont) as previously described (Hamer et al., 1988; Howard et al., 1991a). Briefly, 200 µL of a conidial suspension at a concentration of 105 mL−1 was placed on the surface of a Teflon coverslip and left in a humid chamber for 12 to 14 hr. The coverslip was then gently inverted and viewed by phase contrast or Nomarski interference microscopy. The frequency of appressorium formation was determined from a sample size of 300 conidia per membrane. Frequencies of appressorial formation were determined and tested for significant departure from the expected wild-type frequency by Chi-square tests (Sokal and Rohlf, 1981). The production of spore tip mucilage was assayed by fluorescein isothiocyanate-concanavalin A staining (Hamer et al., 1988). Conidial germination assays were carried out on polystyrene coverslips using conidial suspensions at a concentration of 103 mL−1. Estimations of conidial production were carried out by flooding the surface of plates, containing single 4-cm-diameter mycelial colonies, with 2 mL of sterile distilled water and determining conidial concentrations by haemocytometer counting.

DNA Sequencing

A 2.7-kb BamHI-XbaI fragment containing the MPGl gene was subcloned and designated p15GXB. Nested deletions were generated by exonuclease III digestion (Henikoff, 1984) throughout the genomic subclone p15GXB and the cDNA clone pNJT-15, and DNA sequence information was obtained from both strands using the dideoxy chain termination method (Sanger et al., 1977) using Sequenase (United States Biochemical Corp.). DNA sequence analysis was performed using the Sequence analysis software, version 7.2, of the Genetics Computer Group (GCG) of the University of Wisconsin (Devereux et al., 1984) made available by the AIDS Center Laboratory for Computational Biochemistry at Purdue University. The screening of DNA sequence data bases was performed using the BLAST algorithm (Altschul et al., 1990) at the NCBI or the GCG program FASTA. Amino acid alignments were made by eye and by using BESTFIT, ALIGN, and PILEUP programs of the GCG package. Kyte-Doolittle hydropathy plots (Kyte and Doolittle, 1982) were constructed with a window size of seven, using the program DNA Strider Version 1.0 (Marck, 1988).

Primer Extensions

The 5' end of the MPGl transcript was determined by primer extension using total RNA from nitrogen-starved cultures of M. grisea (see below). The oligonucleotide 5'-GCCAGGATCCACGCTGTGAGGA-GAATCCTG-3' is complementary to the MPGl mRNA for 24 nucleotides starting four nucleotides upstream of the ATG initiation codon. The primer was end labeled with γ-32P-ATP using T4 polynucleotide kinase according to standard protocols (Sambrook et al., 1989). Labeled primer (105 cpm) was annealed to 10 µg of RNA and coprecipitated with 0.1 volume of 3 M sodium acetate and 2.5 volume ethanol. The RNA-oligonucleotide mixture was resuspended in 20 µL of RNase-free distilled water and incubated at 65°C for 5 min. Five microliters of avian myeloblastosis virus reverse transcriptase reaction buffer was added (final concentrations: 50 mM Tris-HCl, pH 8.3, 50 mM KCl, 10 mM MgCl2, 0.5 mM spermidine, 10 mM DTT, 4 mM PPI, and 1 mM each of dTTP, dGTP, and dCTP). The reaction mixture was moved from 65 to 42°C for 5 min and then to room temperature for 5 min to complete annealing of the primer to the MPGl mRNA. Finally, 25 units of RNasin (Promega) and 10 units of AMV reverse transcriptase (Gibco-Bethesda Research Laboratories) were added, and the reaction was incubated for 60 min at 42°C. The primer extension products were analyzed by electrophoresis on an 8% (w/v) urea polyacrylamide sequencing gel and visualized by autoradiography (data not shown). DNA sequencing ladders were used as size standards to estimate the size of the reverse transcribed products.

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