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Genetic Analysis of Developmental Mutants and Rapid Chromosome Mapping of *APP1*, a Gene Required for Appressorium Formation in *Magnaporthe grisea*

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Infection of rice by *Magnaporthe grisea*, the rice blast pathogen, requires formation of a specialized, darkly pigmented, dome-shaped appressorium. To elucidate the mechanism(s) involved in formation of this structure, several developmental mutants were isolated by UV mutagenesis. Two mutants (243-7 and 370-7) with greatly reduced appressorium formation on the hydrophobic surface of GelBond were obtained. An additional mutant, 17-4/3, which was able to form appressoria on the hydrophilic surface of GelBond, was also isolated. A fourth mutant, 138-1, was obtained that produced conidia on complete medium. Genetic analysis of the mutants indicated that the phenotypes are under single gene control. Pairwise crosses between mutants demonstrated that the mutations are at separate loci. The genetic loci in mutants 243-7, 370-7, 17-4/3, and 138-1 were termed *APP1*, *APP2*, *APP3*, and *CCN1*, respectively. From a cross between mutants containing either *app1*$^-$ or *app3*$^-$, two recombinant progeny were obtained from a total of 62, indicating that the two loci are closely linked. The other loci are independent. The addition of cyclic AMP or 1,16-hexadecanediol to the mutant containing *app2*$^-$ restored the ability to form appressoria, but was ineffective on the *app1*$^-$ mutant. DNA bulked segregation analysis from a cross with mapping strain 2539 and restriction fragment length polymorphism markers from genetic maps of *M. grisea* were used to localize the *APP1* locus to a central region of chromosome 2. Fine mapping resulted in the identification of flanking markers cos94 and A14B10 located 0.5 cM on either side of *APP1*, as well as several co-segregating cosmid markers, 4-10, cos91, cos167, and A14D8. The results indicate the utility of linkage maps for efficiently positioning genes of interest in the *M. grisea* genome and should expedite gene isolation by positional cloning.

Additional keywords: genetic crosses, infection-related morphogenesis.

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Magnaporthe grisea (T. T. Hebert) Yaegashi and Udagawa causes rice blast, one of the most devastating diseases on rice (*Oryza sativa* L.) worldwide. Successful infection depends upon formation of a dome-shaped, highly melanized appressorium. Differentiation of this specialized cell from the tip of an emerging germ tube is a response to environmental stimuli. The fate of the germ tube tip depends on the nature of the surface on which it is growing (Hamer et al. 1988; Lee and Dean 1993). On a hydrophilic surface, such as a clean glass microscope slide, the germ tube typically continues to extend and eventually forms a mycelium. However, on other surfaces, such as a rice leaf or the hydrophobic surface of GelBond, hyphal extension ceases and the tip swells to form an appressorium.

M. grisea is well suited for biochemical and genetic investigation into the mechanisms controlling appressorium formation. The fungus is a haploid, heterothallic Ascomycete (Rossman et al. 1990; Valent and Chumley 1991) and highly fertile laboratory strains have been developed (Kolmer and Ellingboe 1988; Leung et al. 1988, Valent et al. 1986; Chao and Ellingboe 1991). Sexual compatibility is governed by alternate alleles of the mating-type locus, *Mat1*; the mating-type genes have recently been cloned (Kang et al. 1994). Other useful features include small genome size, extensive genetic maps, and well-established transformation systems.

The mechanism(s) of surface recognition by the germ tube tip are beginning to be elucidated. Germ tubes of the bean rust fungus, *Uromyces appendiculatas* (Pers.:Pers.) Unger, respond to ridges such as those formed by the guard cell surrounding a stomatal opening (Hoch et al. 1987). Contact with the ridge causes an indentation in the germ tube, resulting in the disorganization of the cytoskeleton from its longitudinal axis (Kwon et al. 1991). It has been suggested that the inability of the microtubules to continue polymerizing beyond this indentation acts as the primary signal receptor, triggering appressorium formation. *M. grisea* does not respond to such topographical signals (Lee and Dean 1994); primary signals include the hydrophobicity and the hardness of the contact surface, as well as molecules from the plant surface (Gilbert et al. 1996). The mechanism(s) involved in surface recognition are unknown; however, progress has been made on elucidating the mechanism(s) of signal relay within *M. grisea*. It was also found recently in our laboratory that cutin monomers, in particular 1,16-hexadecanediol, induce appressorium formation on a hydrophilic surface to a very high level (Gilbert et al. 1996).

We have previously shown that cyclic AMP (cAMP), an important intracellular mediator, is central to infection structure formation in *M. grisea* (Lee and Dean 1993). cAMP typically activates protein kinase activity by binding to and releasing the regulatory subunit from the catalytic subunit (Taylor 1989). Most recently, we have shown that cAMP-dependent protein kinase is involved in appressorium formation. Targeted deletion of *cpkA*, the catalytic subunit of the kinase, affected the ability to form appressoria and to infect susceptible rice plants (Mitchell and Dean 1995).

In order to further investigate mechanism(s) regulating appressorium formation and other aspects of development, we report here the isolation and genetic characterization of four *M. grisea* mutants. The effects of cAMP and 1,16-hexadecanediol on restoring appressorium formation in these mutants are presented. Genetic crosses and allelism tests demonstrate that these mutants are affected at separate loci. Furthermore, we describe the rapid identification of restriction fragment length polymorphism (RFLP) markers tightly linked to one of these genes, *APP1*, using bulked pools of DNA from a segregating F_1 population. Linked markers are further analyzed on DNA from individual progeny to construct a high resolution map of the *APP1* locus on chromosome 2.

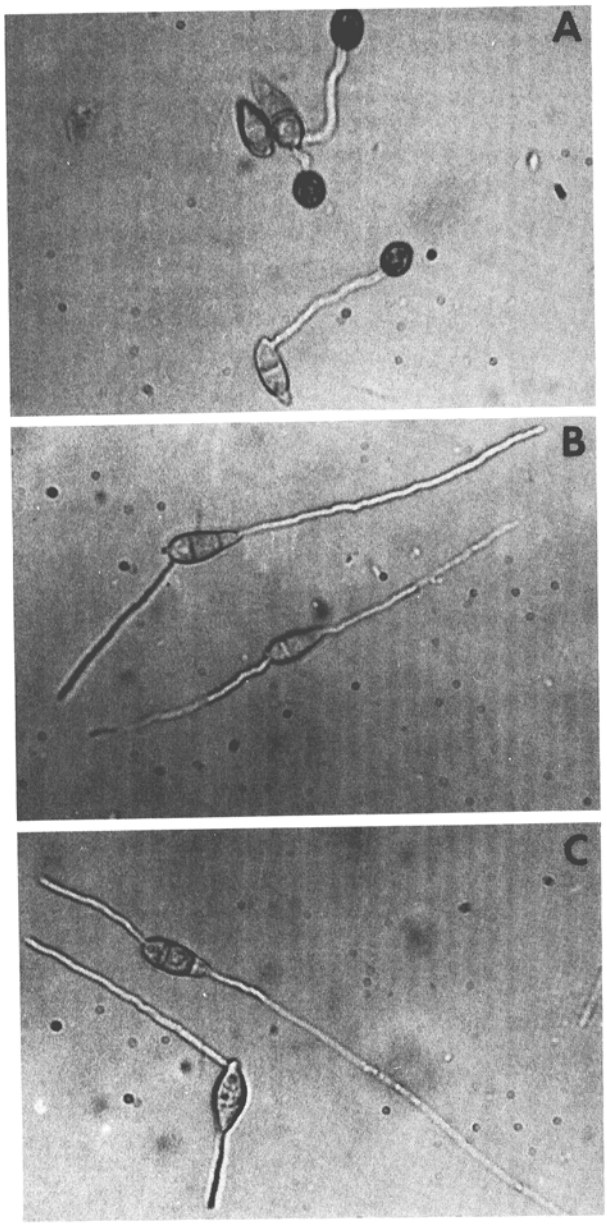

Fig. 1. Appressorium formation by *Magnaporthe grisea* wild-type strain 70-15 (1A), mutant 243-7 (*app1*$^-$, 1B), and 370-7 (*app2*$^-$, 1C) on a hydrophobic surface (GelBond, ×400 magnification). Conidia were collected in water from V8 plates and adjusted to 10^4/ml. The appressorium formation was determined at 8 h after application to the surface.

RESULTS

Isolation and description of mutants.

To obtain appressorium-deficient mutants of *M. grisea*, UV survivors from strain 70-15 were screened for their ability to form appressoria on artificial surfaces. Survivors were allowed to undergo one asexual cycle prior to screening for appressorium formation. Figure 1A shows typical wild-type appressorium formation. Initial screening of 1,500 UV survivors yielded numerous potential mutants. After extensive subculturing on complete media and reevaluation of phenotype 16 auxotrophs, six stable mutants affected in appressorium formation, a germination-deficient mutant, and a mutant that conidiates abundantly on several media were obtained. Three mutants (149-7, 209-7, and 232-7) that were unable to form appressoria on a hydrophobic surface were not characterized further, because they showed complex segregation patterns upon genetic analysis (data not shown). Strains used in this study are described in Table 1.

Appressorium formation by monoconidial isolates of the deficient mutants chosen for further investigation is shown in Figure 1B and C. Mutant 243-7 failed to form appressoria on hydrophobic or hydrophilic surfaces. No appressoria were observed after 24 h of incubation. Appressorium formation by mutant 370-7 was greatly reduced (7%). Both mutants appeared very similar, and produced long, straight germ tubes when germinated on hydrophobic surfaces. Mutant 17-3/4 formed appressoria (93%) on the hydrophobic surface of GelBond indistinguishable from that of wild type (95%), but unlike wild type also formed appressoria (66%) on the hydrophilic surface. A further interesting mutant was also discovered during the screening procedure. Mutant 138-1 was found, unlike the wild type, to conidiate abundantly on CM plates ($6.6 \pm 0.7 \times 10^3$ conidia/cm^2 after 5 days). This mutant also had a slightly reduced ability to form appressoria (70%) on the hydrophobic surface of GelBond. Conidia of the four mutants described above germinated at a rate indistinguishable from the wild type (>90%). The color and colony morphology of the mutants were similar to wild type, with the exception of 138-1, which appeared darker due to conidiation.

Table 1. *Magnaporthe grisea* strains used in this study

Strain	Description	Source
2539	MAT1-1, nonpathogenic on rice	Lung et al. 1988
70-15	MAT1-1, lab strain, pathogenic on rice	Chao et al. 1991
70-6	MAT1-2, lab strain, pathogenic on rice	Chao et al. 1991
243-7	MAT1-1, *app1*$^-$ mutant of 70-15	This study
370-7	MAT1-1, *app2*$^-$ mutant of 70-15	This study
17-4/3	MAT1-1, *app3*$^-$ mutant of 70-15	This study
138-1	MAT1-1, *cnn1*$^-$ mutant of 70-15	This study

Other characteristics, including the size of conidia, the number of cells per conidium, conidiation, and growth rate are presented in Table 2. With the exception of 138-1, conidia of the mutants were similar to wild type. Unlike the three-celled conidia of wild type, conidia of 138-1 were predominantly two celled. The radial growth rate and conidiation of the mutants were significantly reduced compared with wild type.

Genetic crosses and allelism tests.

The results of genetic crosses for the four mutants are presented in Table 3. F_1, backcross, and pooled segregation data provide strong evidence for single genetic loci controlling the phenotypes exhibited by mutants 243-7, 17-4/3, and 138-1. This conclusion is supported by the fact that no wild-type offspring were obtained from sibcrosses between mutant progeny (110S, 303S, 317S, and 401S). F_1 and backcross data for mutant 370-7 also showed a good fit to 1:1 ratio, as does the pooled segregation data, suggesting that this mutant is under single gene control.

To determine the genetic relationships among 243-7, 370-7, 17-4/3, and 138-1, allelism tests were conducted. From pairwise crosses wild-type recombinant progeny were observed, demonstrating that all four mutations are affected at separate loci (Table 4). We conclude that the phenotypes of mutants 243-7, 370-7, 17-4/3, and 138-1 are under single gene control, designated *APP1*, *APP2*, *APP3*, and *CNN1*, respectively. Only two of 62 offspring from the cross between 243-7 and 17-4/3 exhibited a wild-type phenotype, indicating that *APP1* and *APP3* are closely linked. In the remaining crosses, about 25% of the progeny were wild type, as expected for independent loci. For cross 370-7 × 138-1, only three progeny out of 18 had the wild-type phenotype, suggesting possible linkage between *APP2* and *CCN1*. No attempt was made to score double mutants in any cross.

Mapping of *APP1*.

Several genetic maps are available for *M. grisea*, including a map created between Guy11 and the goosegrass isolate 2539 (Skinner et al. 1993). Since the *app1*$^-$ mutation was created in strain 70-15, a direct descendent of Guy11, it was reasoned that the map information from Guy11 × 2539 would be applicable to a cross between the mutant carrying *app1*$^-$ and 2539. Fourteen RFLP markers, distributed over the seven chromosomes, separated by about 40 cM, were shown to be polymorphic between the *app1*$^-$ mutant and 2539. DNA, pooled from 14 mutant progeny and separately from 16 wild-type progeny, were surveyed for RFLPs with these markers. Two markers on chromosome 2, CH3-24H and 4-10, exhibited the predicted RFLP patterns. Based on information provided from an integrated genetic map of *M. grisea* (S. A. Leong and B. Valent,

Table 2. Characteristics of wild type (70-15) and mutants of *Magnaporthe grisea*

	Cells/conidium (no.)[v]			Conidium size[w]		Conidiation[x]		Growth rate[y]	
Strains	1	2	3	L	W	8D	12D	CM	PDA
70-15	12.3 a[z]	7.8 a	79.9 a	21.2 a	8.6 ab	34.3 a	68.0 a	3.2 a	3.2 a
243-7	5.5 b	10.1 ab	84.4 a	21.7 a	8.4 b	9.6 c	19.0 c	2.6 b	2.6 b
370-7	6.2 b	23.6 b	70.2 a	20.8 a	8.4 b	8.4 c	7.8 d	2.7 b	2.6 b
17-4/3	8.7 ab	22.0 b	69.3 a	21.7 a	8.9 a	12.5 b	25.6 b	2.4 c	2.1 c
138-1	6.0 b	58.0 c	32.0 b	21.0 a	7.7 c	0.1 d	11.5 d	2.3 c	2.2 c

[v] Percentage of conidia with 1, 2, or 3 cells. The data are the result of three experiments, 50 conidia examined in each.
[w] L and W = length and width, respectively, of conidia in micrometers. The data are the result of three experiments, 20 conidia examined in each.
[x] Oatmeal agar was inoculated with plugs (0.5 cm) taken from the growing edge of a colony on complete medium (CM). After incubation as described in Materials and Methods for 8 days, conidia were harvested in sterile, distilled water (8D). Plates were reincubated and conidia harvested again 4 days later (12D). The data ($\times 10^4$) are the results of two experiments, four plates examined in each.
[y] Plates of CM and potato dextrose agar (PDA) were incubated with plugs as above. Colony diameters (cm) were measured after 5 days. The data are the results of two experiments, four plates examined in each.
[z] Means in each column followed by the same letter do not differ significantly ($P \leq 0.05$) according to one-way analysis of variance.

Table 3. Segregation of random ascospore progeny from crosses between mutants and wild-type strains of *Magnaporthe grisea*[y]

			Segregation		Chi-square[z]	
Cross number	Cross	Generation	M	W	1 locus	2 loci
101	243-7(M) × 70-6(W)	F_1	12	8	0.45	11.27***
102	102(M) × 70-6(W)	Backcross	11	8	0.21	9.28**
110	110(M) × 70-6(W)	Backcross	3	7	0.90	0.01
110S	110-4(M) × 110-10(M)	Sib	12	0	0.00	...
201	370-7(M) × 70-6(W)	F_1	15	21	0.69	4.48
221	221(M) × 70-6(W)	Backcross	24	14	2.13	27.50***
301	17-4/3(M) × 70-6(W)	F_1	11	9	0.05	12.58***
303	303(M) × 70-15(W)	Backcross	5	4	0.00	3.00
317	317(M) × 70-15(W)	Backcross	12	9	0.19	9.92**
303S	303-8(M) × 303-9(M)	Sib	15	0	0.00	...
317S	317-4(M) × 317-6(M)	Sib	15	0	0.00	...
401	138-1(M) × 70-6(W)	F_1	9	13	0.41	2.19
437	437(M) × 70-6(W)	Backcross	11	11	0.05	6.07*
429	429(M) × 70-15(W)	Backcross	12	12	0.04	6.72**
401S	401-20(M) × 401-37(M)	Sib	29	0	0.00	...

[y] M and W represent mutant type and wild type, respectively.
[z] Values calculated with the Yates correction term; *, **, *** significant at $P = 0.05$, 0.01, and 0.001, respectively.

personal communication) additional RFLP markers were surveyed to fine map the *APP1* locus in a segregating population of 182 individuals. For example, marker A14D8, shown in Figure 2, is closely linked to *APP1*. The predicted order of the markers and *APP1*, as determined by MAPMAKER, is shown in Figure 3. Four markers, 4-10, cos91, cos167, and A14D8, co-segregated with *APP1*. Two additional RFLP markers, cos94 and A14B10, were found at a distance of 0.5 cM on either side of *APP1*.

Chemical induction assay.

cAMP, which has been shown to mediate appressorium formation, was tested for its potential to restore this capability to the appressorium-deficient mutants (Lee and Dean 1993) (Table 5). Mutant *app1*$^-$ germinated but was unresponsive and formed no appressoria, even when incubated for 24 h. Mutant *app2*$^-$ was also unresponsive to cAMP on the hydrophilic surface, but formed high levels of appressoria (88%) when germinated on the hydrophobic surface in the presence of cAMP. Mutant *app3*$^-$ treated with cAMP on a hydrophilic surface formed appressoria at levels indistinguishable from those of wild type. Appressorium formation for mutant *cnn1*$^-$ was stimulated from 5 to 67% by cAMP when conidia were germinated on the hydrophilic surface.

To determine the specificity of cAMP to restore appressorium formation, several cAMP analogs, cGMP, adenosine, and AMP were tested for their effects on appressorium induction. Table 6 shows that cAMP and 8-bromo cAMP induced high levels of appressorium formation in mutant *app2*$^-$. 8-bromo cAMP was the most effective, followed by cAMP and N^6-monobutyryl cAMP. N^6,$O^{2'}$-dibutyryl cAMP, an analog used in mammalian systems, did not induce appressorium formation. cGMP induced a low level of appressorium formation, but adenosine and AMP were ineffective.

Recent work from our laboratory has shown that components of the plant cuticle, 1,16-hexadecanediol in particular, are very effective at inducing appressoria in vitro (Gilbert et al. 1996). 1,16-hexadecanediol was ineffective at stimulating appressorium formation by mutant *app1*$^-$ (Table 5). No appressoria were observed after 24 h of incubation. However, unlike cAMP, it was effective at inducing appressorium formation in mutant *app2*$^-$, germinated on a hydrophilic surface to a fairly high level (58%). Mutant *cnn1*$^-$ was responsive to 1,16-hexadecanediol on the hydrophilic surface, but appressorium formation (63%) was not as high as with the wild type. Appressorium formation for mutant *app3*$^-$ was stimulated from 66 to 78% by 1,16-hexadecanediol on the hydrophilic surface.

Table 4. Genetic relationships between developmental mutations of *Magnaporthe grisea*

Locus pair	Parents	Genotypes	Progeny (no.) WT[y]	Mutant	χ^2
app1/app3	243-7 × 17-4/3	*app1*$^-$*/APP3* × *APP1/ app3*$^-$	2	60	14.53
app1/ccn1	243-7 × 138-1	*app1*$^-$*/CCN1* × *APP1/ccn1*$^-$	9	34	0.19
app2/app3	370-7 × 17-4/3	*app2*$^-$*/APP3* × *APP2/ app3*$^-$	12	31	0.38
app2/ccn1	370-7 × 138-1	*app2*$^-$*/CCN1* × APP2/*ccn1*$^-$	3	15	0.30
app3/ccn1	17-4/3 × 138-1	*app3*$^-$*/CCN1* × *APP3/ccn1*$^-$	10	30	0.03

[y] Wild type.
[z] Values calculated with the Yates correction term. Significant at 0.001 with 1:3 ratio, 1 df.

DISCUSSION

Phenotypes of the mutants.

UV mutagenesis of *M. grisea* isolate 70-15 yielded three stable developmental mutants primarily affected in appressorium formation and a conidiation mutant. Examination of other characteristics of the appressorium mutants revealed that they were defective in other aspects of growth and development (Table 2). Many genes, when investigated thoroughly, have been shown to have pleiotropic effects. In *M. grisea*, it has been shown that mutations at loci affecting infection-related morphogenesis also affect conidiation, and vice versa. Mutations at *SMO*, which affects the ability of conidia to stick to Teflon, are affected in conidium, appressorium, and ascus morphology. *SMO* mutants also show reduced ascospore viability (Hamer et al. 1989). Several sporulation mutants, particularly *con1*$^-$ and *con7*$^-$, obtained by Shi and Leung (1995), also show a reduced ability to form appressoria.

Genetic analysis.

Based on segregation analysis, the four mutations described in this study (*app1*$^-$, *app2*$^-$, *app3*$^-$, and *cnn1*$^-$) are under single

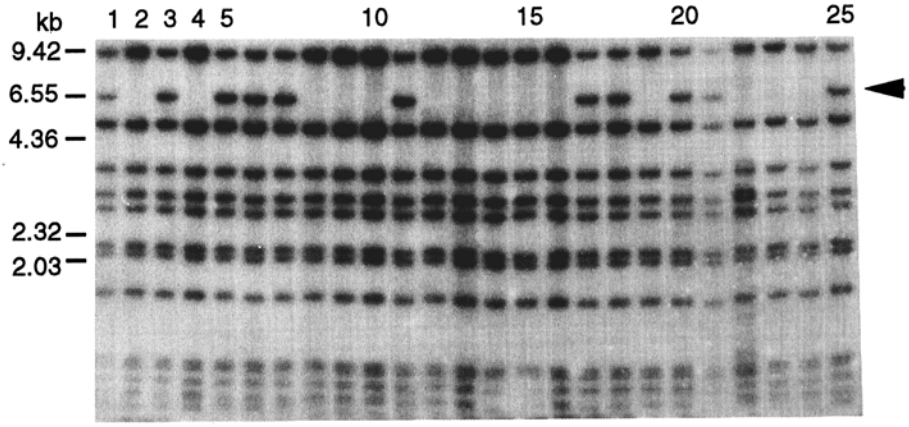

Fig. 2. Marker A14D8 cosegregates with *app1*$^-$. Genomic DNA was digested with *Eco*RI and probed with A14D8. Lanes1, *app1*$^-$ mutant; lane 2, wild type; lane 3, bulked mutant progeny; lane 4, bulked wild-type progeny; lanes 5 to 25, F_1 individuals. Arrow indicates polymorphic band.

gene control. These mutations are highly stable since no reversions to wild type were found during our extensive manipulations. All mutants were fertile but acted predominantly as males in initial backcrosses. Thereafter, all progeny with the exception of mutant *app2*$^-$ behaved as fully fertile hermaphrodites. Multiple attempts at sibcrosses of mutant *app2*$^-$ failed. Perithecia were found rarely, but no asci could be released. It is possible that this mutation also affects certain processes in sexual reproduction (Miller 1990; Hamer and Givan 1990).

Allelism tests confirmed that *app1*$^-$, *app2*$^-$, *app3*$^-$, and *ccn1*$^-$ are separate loci. This result was not unexpected, since each mutant exhibits a different phenotype. We were unable to obtain progeny from crosses between *app1*$^-$ and *app2*$^-$. However, it can be deduced from the available data that they represent different loci: *APP2* and *APP3* are independent, and *APP1* and *APP3* are closely linked; therefore, *APP1* and *APP2* are not allelic. Our finding that *APP1* and *APP3* are closely linked within 4 cM is of potential significance. This will be not only of benefit for the localization of *APP3,* but may identify a region of the genome expressing appressorium-specific genes. There are many examples of functionally related genes being clustered in fungal genomes. In *Aspergillus nidulans*, 19 spore-specific genes are localized to a particular chromosome segment (Gwynne et al. 1984).

Mapping of *APP1*.

The availability of several extensive linkage maps for *M. grisea* and their recent integration greatly facilitated the rapid localization of *APP1* to chromosome 2. Fine mapping of *APP1* resulted in a fairly saturated linkage map around the *APP1* region. We were fortunate that a previously identified, tightly linked cluster of RFLP markers mapped to the *APP1* region (S. A. Leong and B. Valent, personal communication). In our mapping population, recombinant individuals were found among these markers, although several still cosegregated (Fig. 3). Several of these RFLP markers are cosmid clones carrying the hygromycin resistance gene cassette as a selectable marker. These cosmids will not only be valuable for creating a contig of this region, but will be used for positional cloning of the *APP1* gene by complementing the mutant phenotype. Positional cloning has a greater likelihood of success for cloning appressorium-specific genes compared with attempting to complement with entire cosmid libraries. Complementation of appressorium mutants with libraries requires screening many thousands of transformants with time-

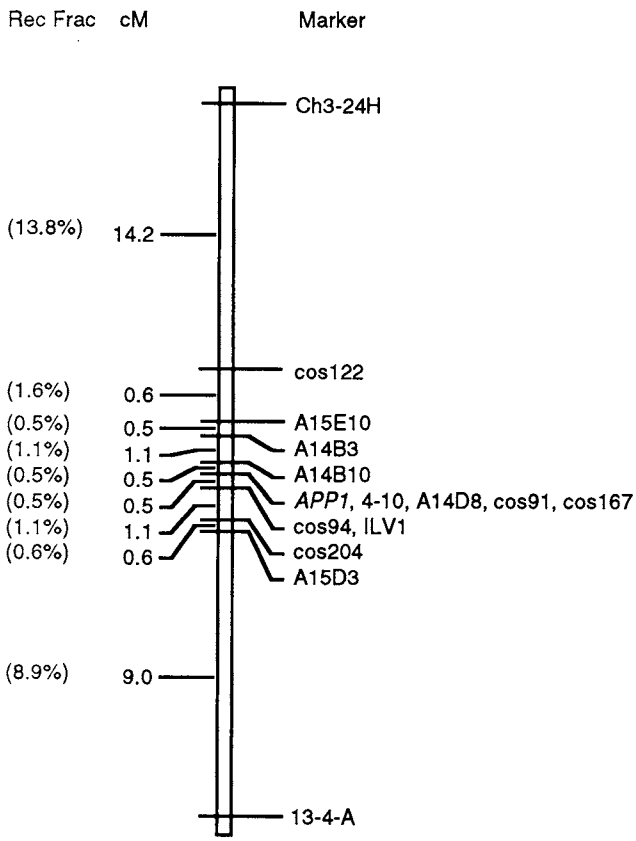

Fig. 3. Genetic map location of *APP1* on chromosome 2 of *Magnaporthe grisea*. The map was created by MAPMAKER with the Kosambi mapping function.

Table 5. Induction of appressorium formation by cAMP and 1,16-hexadecanediol[x]

Strain	Genotype	Hydrophobic surface			Hydrophilic surface		
		H_2O	+cAMP	+diol	H_2O	+cAMP	+diol
70-15	WT[y]	95 ± 0%	ND[z]	ND	5 ± 2%	90 ± 5%	95 ± 0%
243-7	*app1*$^-$	0 ± 0%	0 ± 0%	0 ± 0%	0 ± 0%	0 ± 0%	0 ± 0%
370-7	*app2*$^-$	7 ± 3%	88 ± 8%	89 ± 2%	0 ± 0%	3 ± 2%	58 ± 11%
17-4/3	*app3*$^-$	93 ± 2%	ND	ND	66 ± 19%	95 ± 0%	78 ± 11%
138-1	*ccn1*$^-$	70 ± 10%	93 ± 3%	95 ± 2%	5 ± 2%	67 ± 6%	63 ± 4%

[x] Conidia of each strain were harvested and adjusted to a concentration of 10^4 conidia/ml in water. Cyclic AMP (cAMP) or 1,16-hexadecanediol was added to a final concentration of 10 mM or 1 μM, respectively. Since the stock of 1,16-hexadecandiol was in ethanol, an equivalent volume of ethanol was added to the water control. Appressorium formation was determined as described in Materials and Methods and assessed after 6 h. Results are combined data of at least three experiments with three replicates per experiment, and over 100 conidia examined per replicate.

[y] Wild type.

[z] Not determined.

Table 6. Appressorium formation of mutant 370-7 as affected by cyclic nucleotides, adenosine, AMP, IBMX, and cyclic AMP (cAMP) analogs

Treatment	Germinated conidia that formed appressoria (% ± SD)[z]
cAMP	44.1 ± 15.1
8-bromo cAMP	51.8 ± 6.4
N^6-monobutyryl cAMP, 1 mM	17.1 ± 12.7
N^6,$O^{2'}$-dibutyryl cAMP	2.2 ± 2.1
cGMP	17.8 ± 6.2
AMP	0
Adenosine	0
IBMX, 2.5 mM	43.9 ± 22.1
dH_2O	1.2 ± 1.6

[z] Conidia of mutant 370-7 were harvested and adjusted to a concentration of 10^4 conidia/ml. Conidium droplets (45 μl), plus the appropriate addition (5 μl of stock), were placed on the hydrophobic side of GelBond membrane and incubated in a humid environment at 25°C. All chemicals were used at a final concentration of 10 mM, unless otherwise indicated. Appressorium formation was assessed after 8 h. Results are the combined data of four experiments with three replicates per experiment, with at least 100 conidia examined per replicate.

consuming bio-assays, with no guarantee of success. The cloning of *SMO* with library complementation has been highly problematical (Hamer and Givan 1990).

Chemical induction assays.

Examination of rice leaves has revealed that components of cutin from the leaf surface induce appressorium formation by *M. grisea* (Gilbert et al. 1996). Specific components of the cuticle, including a minor component, 1,16-hexadecanediol, have been shown to be very effective at inducing appressorium formation (Gilbert et al. 1996). This component induced appressorium formation in mutant *app2*$^-$ on a hydrophilic surface, but was not effective at stimulating mutant *app1*$^-$ on either hydrophobic or hydrophilic surfaces. These findings are consistent with the results of allelism tests that demonstrated *APP1* and *APP2* are two different loci. Mutants *app1*$^-$ and *app2*$^-$ were also clearly distinguishable based on response to cAMP. cAMP was not effective at stimulating appressorium formation in either mutant *app1*$^-$ or *app2*$^-$ on hydrophilic surfaces, but was effective for mutant *app2*$^-$ on hydrophobic surfaces. The ineffectiveness of cAMP and 1,16-hexadecanediol on mutant *app1*$^-$ suggests that this mutant may have a lesion in a structural gene responsible for appressorium formation, or in a gene in the signaling pathway downstream of cAMP-dependent protein kinase, or in other regulatory genes.

A number of explanations may account for the ability of cAMP to restore appressorium formation to *app2*$^-$ mutants on a hydrophobic surface only. The mutation may indirectly influence cAMP levels in cells, or be in a gene directly involved in the accumulation of cAMP. The reduced effectiveness of N^6-monobutyryl cAMP on mutant *app2*$^-$ may indicate a mutation in the gene encoding the regulatory subunit of cAMP-dependent protein kinase, reducing the affinity of the mutant protein for cAMP and certain of its analogs. On the other hand, the mutation may increase the affinity of the catalytic and regulatory subunits to each other. Consequently, exogenous addition of the nucleotide is required to induce appressorium formation even on a hydrophobic surface. Alternatively, the *app2*$^-$ mutation may result in the over-expression of phosphodiesterase, causing rapid degradation of cAMP in the cell before triggering appressorium formation. Phosphodiesterase activity levels have been shown to regulate other developmental processes in fungi, such as entry into meiosis in *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae* (Matsumoto et al. 1983; Mochizuki and Yamamoto 1992). Isolation of the affected genes will ultimately lead to the identification of the underlying mechanisms.

MATERIALS AND METHODS

Fungal isolates and cultural conditions.

M. grisea isolates 70-15 and 70-6, used as wild-type controls, were kindly provided by A. Ellingboe (University of Wisconsin, Madison), and mapping strain 2539 by H. Leung (University of Washington). Strains used in this study are shown in Table 1. Media were solidified by the addition of 1.5% agar. Media used for UV survivor isolation were complete medium (CM: yeast extract, 6 g; casein acid hydrolysate, 6 g; sucrose, 10 g; per liter) and potato dextrose agar (PDA; Difco, Detroit, MI) containing 3% sorbose. Auxotrophs were evaluated on minimal medium (MM: $NaNO_3$, 6 g; KCl, 0.5 g; $MgSO_4 \cdot H_20$, 0.5 g; KH_2PO_4, 1.5 g; sucrose, 10 g; trace elements, 1 ml; per liter, pH 6.5). Conidia were produced on V8 medium (V8 juice, 40 ml, pH 7.0, per liter). All cultures were grown under continuous illumination at 21 ± 1°C provided by an equal mixture of fluorescent and blacklight (Sylvania 350, 20W) bulbs.

Mutagenesis and mutant isolation.

Conidia were harvested from 10-day-old cultures in sterile dH_2O, washed by centrifugation, and adjusted to a concentration of 10^5 conidia/ml. The conidia suspension (5 ml) was stirred in a petri dish 10 cm below a UV source (UVP, Minerallight lamp, model UVGL-25). A 5-min exposure killed 95% of the conidia. The irradiated conidia suspension was adjusted to give approximately 60 survivors per plate and was plated on CM plus sorbose to restrict colony growth. Resulting colonies were transferred by toothpick to 24-well microtiter plate wells containing oatmeal agar prepared by heating oatmeal (50 g) in 500 ml of water at 70°C for 1 h, filtering through cheesecloth, and adjusting the volume of the filtrate to 1 liter, or onto petri plates containing CM agar.

Phenotypic characterization on artificial surfaces.

Conidia were harvested from individual wells 7 days later in sterile, distilled water and adjusted to a concentration of approximately 10^4 conidia/ml. Droplets (25 µl) were placed on hydrophobic and hydrophilic sides of GelBond membrane (a hydrophobic polyester sheet produced by FMC, Rockland, ME, with one side coated with a thin layer of agarose to create a hydrophilic surface) at roughly 1-cm intervals supported by moistened paper towels lining a plastic box. The moist chamber was sealed with plastic wrap and incubated for 6 h at room temperature. Subsequent microscopic examination (×400) revealed those mutants unable to form appressoria on a hydrophobic or those able to form appressoria on a hydrophilic surface. Mutant 138-1 and its progeny were grown on CM agar or PDA in petri dishes for 3 to 4 days prior to observing conidium formation under a light microscope.

Segregation analysis and allelism tests.

Crosses were made by placing two strains about 5 cm apart on oatmeal agar in a petri plate, and incubated at 22°C for 14 to 24 days. Individual perithecia were removed, crushed, and dragged across the agar surface. Individual asci were separated from each other with a metal needle, and ascospores allowed to germinate at 22°C overnight. Asci were then transferred to V8 plates, where they produced conidia in about 1 week. Conidia were streaked on CM agar plates and a single conidium was isolated from each ascus colony, thus ensuring the collection of one progeny from each meiotic event.

Each of the four mutants was crossed reciprocally with the wild-type strain. The segregation ratios observed in F_1 progeny were confirmed by backcrosses and sib-crosses. Mutant type F_1 progeny were chosen to backcross with the compatible wild-type strain. Sibcrosses were made between two mutant-type progeny either from F_1 population or from backcross population.

All mutants were tested for allelism by procedures described above. Progeny were tested for segregation of mutant phenotypes. In order to be assigned as a wild-type progeny, an offspring had to fulfill the following criteria: (i) absence of

conidiation on CM in any crosses with *ccn1*$^-$; (ii) absence of appressorium formation on hydrophilic surface in any crosses with *app3*$^-$; and (iii) appressorium formation on hydrophobic surface in any crosses with *app1*$^-$ and/or *app2*$^-$.

DNA markers and manipulation.

RFLP markers for mapping were kindly provided by S. A. Leong and B. Valent. The markers provided by Leong's group were #11, CH3-24H, 4-10, 4-3, 4-14, CH3-123H, 4-178, CH3-44H, CH2-57H, CH5-184H, G131R, CH3-85H, CH5-58H, 13-4-A, 4-89, and 19-10-A. Valent's group provided markers ILV1, A1D5, A14B3, A14B10, A14D8, A15D3, A15E10, cos91, cos94, cos113, cos122, cos167, cos204, cos211, and cos247. Plasmid and cosmid DNA were isolated with the alkaline method of Sambrook et al. (1989). *M. grisea* DNA was isolated as described (Yelton et al. 1984). Restriction digestion, agarose gel fractionation, Southern blot, and RFLP analysis were performed according to the manufacturers' instructions and standard methods (Sambrook et al. 1989). Hybridization was carried out in 6× SSC (1× SSC is 0.15 M NaCl, 0.015 M sodium Citrate), 5× Denhardt's solution (1× Denhardt's solution is 0.02% Ficoll 400, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin), 0.1% sodium dodecyl sulfate (SDS), and 50 mM phosphate buffer, pH 6.6, at 65°C. Membranes were washed down to 0.5× SSC, 01% SDS, before being exposed to X-ray film.

Mapping of *APP1*.

A cross of mutant *app1*$^-$ × strain 2539 was made to produce an F_1 population. Permanent dry stocks of 182 individuals were made by growing each individual on CM, air dried and stored at –20°C. Progeny were grown on V8 media to produce spores and scored for appressorium formation on the hydrophobic surface of GelBond. Segregation data were analyzed by chi square against 1:1 ratio.

For bulked segregation analysis, 1 µg of DNA from 14 mutant progeny was pooled. A second pool was created with DNA from 16 wild-type progeny. Pooled DNA was digested with appropriate restriction enzymes to search for possible RFLPs. Fourteen markers, distributed over the seven chromosomes separated by about 40 cM, were surveyed for RFLPs between the two pools. Fifteen additional RFLP markers were chosen to perform fine mapping based on proximity to the location of *APP1* on chromosome 2. DNA from total of 182 progeny was used in this analysis. Lanes were scored for genotypes (1, 0) at polymorphic loci. A linkage map was created by MAPMAKER Macintosh V2 (Lander et al. 1987).

Chemical induction assay for appressorium formation.

For cAMP assays, the conidial suspension was adjusted to a concentration of 10^4 conidia/ml. Five microliters of a 10× cAMP stock solution (100 mM) was mixed with 45 µl of conidium suspension and added to the hydrophilic surface of GelBond. Conidia were then incubated as described above.

For 1,16-hexadecanediol assays, glass slides were cleaned with 2 N NaOH and rinsed exhaustively with distilled water. Approximately 10^4 conidia were placed on the slide in 2 ml of sterile, distilled water for 1 h before being placed in a 50-ml sterile, conical tube containing 40 ml of sterile, distilled water and 1 M 1,16-hexadecanediol (Gilbert et al. 1996). After 6 h of incubation, percent appressorium formation was determined with a light microscope. Chemical induction assays were performed at least three times in independent experiments with three replicates per treatment.

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