Isolation of the Mating-Type Genes of the Phytopathogenic Fungus *Magnaporthe grisea* Using Genomic Subtraction

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Manuscript received December 13, 1993
Accepted for publication June 13, 1994

ABSTRACT

Using genomic subtraction, we isolated the mating-type genes (*Mat1-1* and *Mat1-2*) of the rice blast fungus, *Magnaporthe grisea*. Transformation of *M. grisea* strains of one mating type with a linearized cosmid clone carrying the opposite mating-type gene resulted in many "dual maters," strains that contain both mating-type genes and successfully mate with both *Mat1-1* and *Mat1-2* testers. Dual maters differed in the frequency of production of perithecia in pure culture. Ascospores isolated from these homothallic crosses were either *Mat1-1* or *Mat1-2*, but there were no dual maters. Most conidia from dual maters also had one or the other of the mating-type genes, but not both. Thus, dual maters appear to lose one of the mating-type genes during vegetative growth. The incidence of self-mating in dual maters appears to depend on the co-occurrence of strains with each mating type in vegetative cultures. In rare transformants, the incoming sequences had replaced the resident mating-type gene. Nearly isogenic pairs produced from three *M. grisea* laboratory strains were mated to investigate their fertility. One transformant with switched mating type appears to have a mutation that impairs the development of asci when its mating partner has a similar genetic background. The *M. grisea Mat1-1* and *Mat1-2* genes are idiomorphs approximately 2.5 and 3.5 kb in length, respectively.

MAGNAPORTHE grisea (Hebert) Barr (anamorph, *Pyricularia grisea* Sacc.) is a haploid, filamentous, heterothallic Ascomycete, order Pyrenomycetes (ROSSMAN *et al.* 1990; VALENT and CHUMLEY 1991). As with other Ascomycetes, compatibility for mating is governed by alternate alleles of the mating-type locus, *Mat1*. Cloning the *M. grisea* mating-type genes is the first step in understanding and manipulating the mating system for this agronomically important family of plant pathogens.

Rigorous genetic analysis of pathogenicity in *M. grisea* is facilitated by using pairs of nearly isogenic strains of opposite mating-type. This is because differences in genetic background can modify the phenotype associated with genes for pathogenicity. In theory, backcrossing regimes can eliminate differences in genetic background between strains of interest. In practice, however, creating interfertile, nearly isogenic strains may be very difficult or impossible (VALENT *et al.* 1991). The easiest way to obtain a nearly isogenic pair of strains of opposite mating type would be to switch the mating-type locus resident in one strain for the alternative allele.

Although fertile laboratory strains have been developed (KOLMER and ELLINGBOE 1988; LEUNG *et al.* 1988; VALENT *et al.* 1986; CHAO and ELLINGBOE 1991; VALENT *et al.* 1991; SILUÉ *et al.* 1992), significant biological questions are not now accessible to genetic analysis due to poor fertility of key field isolates. One factor that limits fertility in *M. grisea* crosses may be differences in genome arrangement between strains. Translocations and other genetic rearrangements appear to be common among field isolates of the fungus (TALBOT *et al.* 1993; M. J. ORBACH, F. G. CHUMLEY and B. VALENT, unpublished results). Production of nearly isogenic strains by gene replacement at the mating-type locus would immediately eliminate problems due to differences in genome arrangement between parental strains. Gene replacement strategies might also permit genetic crosses that are not now feasible due to mutations in the mating-type genes. Substitution of the mating-type gene in a sterile strain of particular interest with the gene from a highly fertile strain may restore fertility, although it is unlikely that defects at the mating-type locus are solely responsible for sterility in all sterile strains (VALENT *et al.* 1991; CHAO and ELLINGBOE 1991).

The mating-type genes have been cloned from other Ascomycetes using several methods. Chromosome walking from a linked gene was used to clone the *MATA* gene of *Neurospora crassa* (GLASS *et al.* 1988). DNA clones carrying the *mat*$^-$ allele of *Podospora anserina* were isolated using the Neurospora *MATA* gene as a hybridization probe (PICARD *et al.* 1991). The *MAT-1* gene of *Cochliobolus heterostrophus*, a maize pathogen, was cloned by screening for a homothallic transformant after transforming a *MAT-2* strain with cosmid clones derived from a *MAT-1* strain (TURGEON *et al.* 1993). Analysis of these mating-type genes revealed that the alternative mating-type loci in each system contain nonhomologous sequences (DEBUCHY and COPPIN 1992; GLASS *et al.* 1990; STABEN and YANOFSKY 1990; TURGEON *et al.* 1993). However, for a given fungal species,

TABLE 1

M. grisea strains used in this study

Strain	Mating type[a]	Origin[b]
O-135	*Mat1-1*	*Oryza sativa*
Guy11	*Mat1-2*	*O. sativa*
WGG-FA40	*Mat1-1*	*Eleusine coracana*
G-71	*Mat1-2*	*E. coracana*
K76-79	*Mat1-2*	*Eragrostis curvula*
4091-5-8	*Mat1-2*	Cross: WGG-FA40 × K76-79
4136-2-2	*Mat1-1*	Cross: 4091-1-6 × 4091-14-3
4136-4-3	*Mat1-1*	Cross: 4091-1-6 × 4091-14-3
CP2732	*Mat1-2*	4136-2-2, switched mating type
CP2735	*Mat1-2*	4136-4-3, switched mating type
CP2738	*Mat1-1*	4091-5-8, switched mating type
CP3000	*Mat1-1, Mat1-2*	4136-4-3, dual mater
CP3001	*Mat1-1, Mat1-2*	4136-4-3, dual mater
CP3002	*Mat1-2, Mat1-1*	4091-5-8, dual mater
CP3003	*Mat1-2, Mat1-1*	4091-5-8, dual mater

[a] Mating type was based on the results of conventional crossing to tester strains and probing with pMAT1 and pMAT2.

[b] Field isolates of the pathogen are identified by the host plant on which the isolate was found. Crosses that generated laboratory strains have been described (VALENT and CHUMLEY 1987; VALENT *et al.* 1987).

TABLE 2

Tetrads from cross 4091 (WGG-FA40 × K76-79)

Ascus	Spore[a]	Mating type
1	2	*Mat1-1*
	3	*Mat1-1*
	5	*Mat1-2*
	6	*Mat1-2*
5	1	*Mat1-1*
	4	*Mat1-1*
	7	*Mat1-2*
	8	*Mat1-2*
10	1	*Mat1-2*
	2	*Mat1-2*
	4	*Mat1-1*
	6	*Mat1-1*
25	1	*Mat1-1*
	2	*Mat1-1*
	3	*Mat1-2*
	5	*Mat1-2*

[a] One progeny from each of the pairs of identical (sister) spores in each ascus was chosen for this study (HEATH *et al.* 1990; VALENT and CHUMLEY 1987; VALENT *et al.* 1986).

sequences flanking the mating-type locus are essentially identical in isolates of different mating type. METZENBERG and GLASS (1990) introduced the term idiomorph to designate unrelated sequences at the same locus.

A strategy for cloning the *M. grisea* mating-type genes based on homology to previously cloned genes was not feasible, because no homology could be detected. We therefore attempted a technique called genomic subtraction, which is a strategy for cloning DNA segments that are present in one genome, but absent from the other (STRAUS and AUSUBEL 1990; SUN *et al.* 1992; WIELAND *et al.* 1990). Our approach was based on an assumption that the two mating-type genes of *M. grisea* would contain nonhomologous sequences as observed in other Ascomycetes. In this report, we describe the successful use of genomic subtraction for cloning mating-type idiomorphs from *M. grisea*. We report fertility characteristics of strains that now contain the opposite mating-type idiomorph, and of dual-mater strains that contain both mating-type idiomorphs.

MATERIALS AND METHODS

Strains, growth conditions, and transformation: Strains of *M. grisea* used in this study are listed in Tables 1 and 2. Nearly isogenic strains of *N. crassa* used for a control experiment are ORS*a* (FGSC no. 2490) and 74-OR23-IV *A* (FGSC no. 2489); "FGSC" indicates Fungal Genetics Stock Center (University of Kansas Medical Center). *Escherichia coli* DH5α (HANAHAN 1983) was used for maintaining plasmids. Two highly fertile *M. grisea* laboratory strains, 4136-4-3 (*Mat1-1*) and 4091-5-8 (*Mat1-2*), were used as tester strains for mating type (VALENT and CHUMLEY 1987; VALENT *et al.* 1986).

Transformation of *M. grisea* was performed as described previously (SWEIGARD *et al.* 1992a) with minor modifications: the hygromycin B (HygB) concentration in the selection medium was increased to 200 μg/ml, and 20% sucrose was substituted for 1 M sorbitol in STC, regeneration top agarose, and selection medium. Gene replacements were performed by a minor variation on the procedure described for disruption of the *M. grisea CUT1* gene (SWEIGARD *et al.* 1992b). The cosmid DNA was digested with the restriction enzyme *Not*I to release the insert from the plasmid DNA before transformation.

Assessing the fertility of *M. grisea* crosses: The fertility of *M. grisea* field isolates ranges from total sterility (inability to mate with any other strain) to full fertility (ability to mate as a male or as a female and produce hundreds of viable asci per perithecium). Proficiency at mating as a male and/or female partner was assessed in crosses on oatmeal agar plates (VALENT *et al.* 1986, 1991). Results were confirmed by at least one additional round of testing.

Viability of ascospores was determined as follows. In a fertile *M. grisea* cross, mature asci exude directly through the perithecial neck wall and immediately release ascospores into a viscous liquid drop on the outer neck surface. Freshly released ascospores were collected using a small inoculating loop containing 0.25% gelatin solution (no. G-0510, Sigma, St. Louis, Missouri). Collected ascospores were rinsed into a 50–200-μl droplet of gelatin solution onto a 2YEG agar plate (VALENT *et al.* 1991), and the drop was spread to separate individual ascospores. The percentage of viable ascospores was determined 24 hr later using a stereomicroscope at 80× magnification. Ascospore germination determined in this manner was confirmed by releasing intact asci from the bulbs of maturing perithecia and spreading them onto the surface of an agar plate. These asci were examined microscopically to determine if some asci had missing or badly deformed ascospores that would not be noticed in the procedure described above. Germination of ascospores within these acsi was also checked. Viable ascospore counts were taken over a period between 13 and 20 days after a cross was initiated.

Nucleic acid analyses: Genomic DNA of *M. grisea* was prepared from mycelia grown in complete medium (VALENT *et al.* 1986) as described (HAMER *et al.* 1989) except that DNA was purified by CsCl gradient centrifugation. Neurospora genomic DNA was prepared as described above except that mycelium was grown in Vogel's medium N (DAVIS and DESERRES 1970). Genomic DNA from transformants and ascospores was prepared by a miniprep procedure (SWEIGARD *et al.* 1990). Southern analysis was performed with Hybond-N membrane as

recommended by the manufacturer (Amersham Corp., Arlington Heights, Illinois). Labeled DNA probes were prepared by random priming (FEINBERG and VOGELSTEIN 1983).

The plasmid pOKE76, containing the Neurospora *a* idiomorph in pGem3Zf(+) (Promega, Madison, Wisconsin), was generously provided by R. L. METZENBERG (University of Wisconsin). Genomic DNA libraries of the *Mat1-1* strain 4392-1-6 (M. J. ORBACH, F. G. CHUMLEY and B. VALENT, unpublished results) and the *Mat1-2* strain 4091-5-8 (A. R. KUBELIK, F. G. CHUMLEY and B. VALENT, unpublished results) were used to isolate cosmid clones containing the mating-type genes of *M. grisea*.

Genomic subtraction: Genomic subtraction was performed as described by STRAUS and AUSUBEL (1990) with minor modifications. *Sau*3AI-digested DNA (0.25 μg) was mixed with 10 μg of biotinylated DNA for the first subtractive hybridization. In subsequent rounds of subtraction, 5 μg of biotinylated DNA was used to subtract the remaining DNA from the previous cycle, and the amount of avidin-coated beads used for removing biotinylated DNA was halved accordingly. We saved 1/10th of the DNA remaining after the third and subsequent rounds of subtraction for amplification by polymerase chain reaction (PCR). DNA capped with *Sau*3AI adaptors was amplified in 50 μl of PCR mixture (80 mM Tris-HCl, pH 9, 20 mM NH_4SO_4, 1.5 mM $MgCl_2$, each dNTP at 0.2 mM, 0.2 μg of a primer, and 1 unit of *Taq* polymerase) using the GeneAmp PCR system 9600 (Perkin-Elmer, Norwalk, Connecticut). The mixture was subjected to 40–45 cycles of amplification (1 min at 94°, 1 min at 55°, and 1 min at 72°) followed by a 4-min extension at 72°. Labeling of the amplified DNA was performed as described previously (STRAUS and AUSUBEL 1990). The amplified DNA was digested with *Sau*3AI and cloned into *Bam*HI-digested pGem3Zf(−).

RESULTS

Cloning of the mating-type genes of *M. grisea*: Since isogenic strains with different mating types were not available in *M. grisea*, we pooled according to mating type the genomic DNAs from two field isolates, K76-79 and WGG-FA40, and individual members of four tetrads derived by crossing these two isolates (Table 2). The pooled DNA of *Mat1-1* strains was biotinylated and subtracted from the pooled DNA of *Mat1-2* strains.

A subtraction experiment designed to enrich for Neurospora *a* sequences was also performed to estimate the degree of enrichment for sequences corresponding to the *M. grisea Mat1-2* gene. This side-by-side control experiment was useful in monitoring progress with each subtraction step. PCR amplification products obtained after five rounds of subtraction were labeled and used as a hybridization probe on a genomic DNA blot containing digested DNA of Neurospora *A* and *a* strains. As shown in Figure 1, the amplification products hybridized to DNA fragments specific for the Neurospora *a* strain. To make a quantitative estimate of enrichment for DNA sequences of the *N. crassa a* idiomorph, we cloned the amplified DNA into pGem3Zf(−) and performed a colony hybridization with labeled DNA containing the *a* idiomorph. Based on a formula from WIELAND *et al.* (1990), we estimate that we obtained 3000-fold enrichment of *a* sequences after four rounds of subtraction and 3600-fold enrichment after one more round of subtraction.

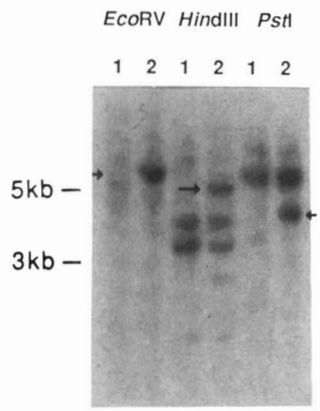

FIGURE 1.—Southern analysis of Neurospora genomic DNA using amplified DNA from genomic subtraction as a probe. Genomic DNA from a Neurospora *A* strain (lanes marked "1") and a Neurospora *a* strain (lanes marked "2") was digested with *Eco*RV, *Hin*dIII and *Pst*I, as indicated. The blot was probed with amplified DNA obtained after five rounds of enrichment for sequences specific to the *MAT a* idiomorph. An arrow indicates the DNA fragment carrying the Neurospora *a* idiomorph. The Neurospora *MAT***a** idiomorph, contained in a 3.3-kb *Pvu*II-*Sph*I fragment from pOKE76, was labeled and shown to hybridize to the same-size, *a*-specific fragments identified by the amplification product (not shown). The amplified DNA used to probe this blot appeared to contain rDNA sequences, because those bands present in the *Hin*dIII and *Pst*I digests of both *A* and *a* strains correspond to rDNA. Their hybridization intensity was comparable to that of the bands containing the *a* idiomorph simply because there are more than 100 copies of the rDNA in *N. crassa* (BUTLER and METZENBERG 1989). Size standards included in the experiment were the 1-kb Ladder Standards (BRL, Gaithersburg, Maryland).

After four rounds of subtraction with *M. grisea* DNA, we cloned the amplified DNA into pGem3Zf(−) and screened individual clones for those that contained sequences that co-segregated with the mating-type locus. Among 30 clones screened, 10 contained inserts that hybridized to genomic sequences found only in *Mat1-2* strains. However, analysis with a larger segregating population, previously used in construction of a genetic map of *M. grisea* (SWEIGARD *et al.* 1993), revealed that the majority of the clones (8/10) came from a locus tightly linked to, but not identical to the mating-type locus (seven recombinants were identified among the 67 members of the mapping population). One clone, called pMAT2, contained a 300-bp *Sau*3AI fragment that hybridized to genomic DNA from all *Mat1-2* strains in the mapping population and failed to hybridize to genomic DNAs from all *Mat1-1* strains (Figure 2).

To determine if the 300-bp *Sau*3AI fragment in pMAT2 corresponded to the *Mat1-2* gene, we used this DNA fragment as a probe to screen a cosmid library from a *Mat1-2* strain, 4091-5-8. Three different overlapping cosmid clones were isolated. These cosmids were used as donor DNA in transformation experiments with the

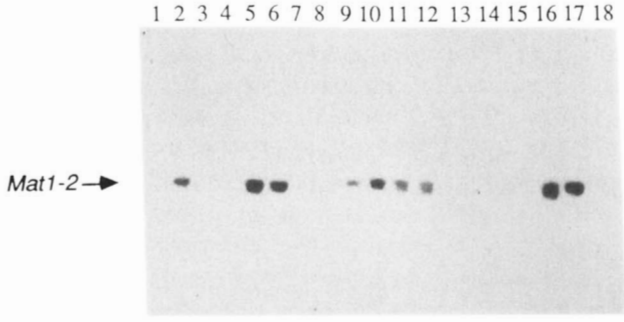

FIGURE 2.—Southern analysis of genomic DNA from members of four tetrads of cross 4091 and their parents. The 300-bp *Sau*3AI insert from pMAT2 (see text) was used as a hybridization probe. Genomic DNA was digested with *Eco*RI: lane 1 (WGG-FA40), lane 2 (K76-79), lanes 3 through 18 (four tetrads in order as in Table 2).

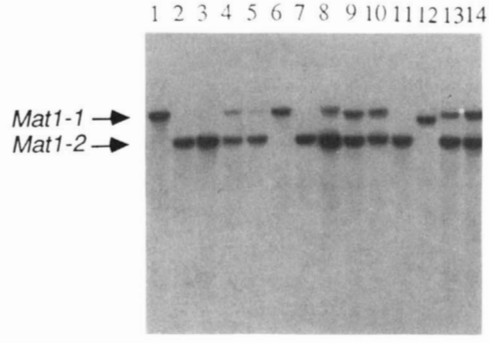

FIGURE 3.—The construction of isogenic strains and dual maters by transformation. Southern analysis was performed on genomic DNAs digested with *Pst*I. The 8-kb *Pst*I fragment containing the *Mat1-2* gene was used as the hybidization probe. Lane 1 contained DNA from 4136-2-2 (*Mat1-1*), lane 6 contained DNA from 4136-4-3 (*Mat1-1*) and lane 11 contained DNA from 4091-5-8 (*Mat1-2*). Strains containing transgenes were transformants of 4136-2-2, lanes 2–5; transformants of 4136-4-3, lanes 7–10; transformants of 4091-5-8, lanes 12–14. Transformants shown in lanes 2, 3, 7 and 12 mated as strains with a switched mating type and those in lanes 4, 5, 8, 9, 10, 13 and 14 mated as dual maters. Size standards included in the experiment were the 1-kb Ladder Standards.

Mat1-1 strain 4136-4-3 as a recipient. One cosmid, pCMT2, consistently yielded transformants that performed as dual maters, strains that produced perithecia and viable ascospores in crosses with both *Mat1-1* and *Mat1-2* strains. To identify cosmid clones containing the *Mat1-1* gene, the 300-bp *Mat1-2*-specific *Sau*3AI fragment was shown to hybridize to an 8-kb *Pst*I fragment of the *Mat1-2* containing cosmid, pCMT2. This *Pst*I fragment was used as a hybridization probe to identify cosmid clones containing the *Mat1-1* gene in another library constructed with genomic DNA of a *Mat1-1* strain, 4392-1-6. One such cosmid, pCMT1, converted 4091-5-8 into a dual mater when introduced into 4091-5-8 by transformation. Thus, both mapping data and functional tests indicated that we cloned the *Mat1-1* and *Mat1-2* genes of *M. grisea*.

The 8-kb *Pst*I fragment from the *Mat1-2* cosmid, pCMT2, corresponded to a 10-kb *Pst*I fragment in the identified *Mat1-1* cosmid, pCMT1. Four unique *Sau*3AI fragments specific to the *Mat1-1* locus were isolated from this 10-kb *Pst*I fragment. One of these, a 250-bp *Sau*3AI fragment, was used as the *Mat1-1* specific fragment, pMAT1.

Construction of isogenic pairs of strains that differ in mating type: Transformants selected for HygB resistance were screened by crossing them with individual mating-type tester strains. Three laboratory strains (4136-2-2, 4136-4-3, 4091-5-8) and O-135 (*Mat1-1*), a female-sterile field isolate that infects rice, were used as recipients in transformation. Rare transformants had switched mating type (Figure 3) and mated only with strains of the same mating type as the original recipient strain. The frequency of gene replacement at the mating-type locus varied depending on the recipient strain. Among 27 HygB-resistant transformants of 4136-4-3 (*Mat1-1*), we found three *Mat1-2* transformants and 12 dual maters. Among 27 transformants of 4091-5-8 (*Mat1-2*), we found one *Mat1-1* transformant and three dual maters. Three *Mat1-2* transformants and five dual maters were identified among 36 transformants of 4136-2-2 (*Mat1-1*). Repeated attempts to find transformants of O-135 with switched mating type yielded only a few dual maters, probably because of the relatively low level of fertility in this strain. Dual maters and strains with switched mating type showed repetitive DNA fingerprinting profiles (HAMER *et al.* 1989) identical to those of the original recipient strains (data not shown), thus eliminating contamination as a possibility for their origin.

Analysis of transformants with switched mating type: We switched the mating type of three laboratory strains to investigate the effect on fertility of these strains. Strains 4091-5-8 and 4136-4-3 mate reproducibly to yield hundreds of perithecia, with hundreds of asci per perithecium and 95% or greater viability of ascospores. Strain 4136-2-2 is a sibling of 4136-4-3 (VALENT and CHUMLEY 1987) that is less proficient at forming perithecia than 4136-4-3. Although the numbers of asci produced are comparable when both strains are crossed with 4091-5-8, only 10–20% of the progeny are viable in the cross with 4136-2-2 (Table 3). One possible explanation is that differences in chromosome structure between 4136-2-2 and 4091-5-8 lead to ascospores with genetic duplications or deletions, resulting in death of the ascospores.

A cross between 4136-4-3 (*Mat1-1*) and CP2735 (4136-4-3 switched to *Mat1-2*) produced hundreds of asci per perithecium, and the viability of ascospores was >90%. The numbers of perithecia produced, numbers of asci per perithecium and viability of ascospores were all comparable to results from the standard control cross between 4136-4-3 and 4091-5-8 (Table 3). When we measured the viability of ascospores from a cross

TABLE 3

Viability of ascospores and perithecia formation in crosses with transformants that have switched mating type

Mat1-2 parent[a]	*Mat1-1* parent[a]	Perithecia formation[b]	Percent viable ascospores
4091-5-8	4136-4-3	++++	95
4091-5-8	4136-2-2	++	10–20
CP2732	4136-2-2	+	10
CP2735	4136-4-3	++++	>90
4091-5-8	CP2738	++++	No asci

[a] The parental strains arose through crosses or by transformation (see Table 1 and RESULTS).

[b] The symbols represent the relative numbers of perithecia formed, with ++++ representing the maximum numbers ever seen.

between 4136-2-2 (*Mat1-1*) and a "switched" version of the same strain (CP2732; *Mat1-2*), we did not observe a significant increase in ascospore viability as compared to the cross between 4136-2-2 and 4091-5-8 (Table 3). This result suggests that the low viability of ascospores in crosses involving 4136-2-2 is not due to aberrant chromosome structure. Few perithecia were formed in this cross, but the number of asci in a single perithecium was comparable to that of highly fertile crosses. Crosses with the other two 4136-2-2 transformants with switched mating type showed the similar results (data not shown).

A transformant of 4091-5-8 (named CP2738) in which the mating-type gene was switched to *Mat1-1* produced abundant perithecia in crosses with 4091-5-8 (Table 3). However, these perithecia contained no asci. Further analysis showed that crosses of CP2738 with another *Mat1-2* strain, K76-79, also produced abundant empty perithecia, and the rare perithecia that contained asci had relatively low numbers. In contrast, all perithecia produced when CP2738 was mated with two other strains, Guy11 or G-71, were filled with asci. The *Mat1-2* strains that were mated with CP2738 were chosen for their high fertility in crosses with strain 4136-4-3 (hundreds of perithecia produced and each perithecium filled with hundreds of asci). All four crosses involving CP2738 were comparable to crosses with 4136-4-3 in perithecial development. Thus, in some, but not all crosses, CP2738 appears to carry a defect in the production of asci.

Analysis of the dual maters: Dual maters were equally fertile when crossed with either *Mat1-1* and *Mat1-2* strains. However, the frequency of perithecia produced by these dual maters as homothallic strains ranged from very few perithecia developed per plate to plates of fungus densely covered with perithecia. Even among dual maters derived from the same strain, the densities of perithecial development differed significantly. In all cases tested, perithecia that did develop contained normal numbers of asci.

CP3000, a dual mater derived from 4136-4-3, was so fertile that the whole plate was completely covered with perithecia. In contrast, CP3001, another dual mater from 4136-4-3, only produced small patches of perithecia surrounded by large sterile sectors. The number of asci in a single perithecium and the ascospore viability were indistinguishable between the two self-crosses. Surprisingly, the mating type of all ascospores that were tested, 20 for CP3000 and 16 for CP3001, was either *Mat1-1* (11 and 10, respectively) or *Mat1-2* (9 and 6, respectively), but there were no dual maters. Results from Southern analysis confirmed that these ascospores contained homology to either *Mat1-1* or *Mat1-2*, but not to both (data not shown). DNA fingerprinting profiles of these ascospores were identical to that of 4136-4-3, eliminating contamination as an explanation for their origin. Results from analysis of the mating type of conidia from both strains strongly suggested that deletion of one mating-type gene occurred before the sexual cycle. Ten random conidia of CP3000 were equally divided among *Mat1-1* and *Mat1-2*, but there were no dual maters. However, the majority of 20 conidia isolated from CP3001, 10 conidia (1 *Mat1-1*, 8 *Mat1-2*, and 1 dual mater) isolated from a sector where perithecia formed and 10 (all *Mat1-2*) isolated form a sterile sector, were *Mat1-2*. These results suggested that deletion of one mating-type gene occurs extremely frequently in these dual maters and that the relative ratio of resulting mating types in the vegetatively growing colony appears to determine the fertility.

Conidia of two dual maters of 4091-5-8, CP3002 and CP3003, which produce only a few perithecia in self-crosses, were also analyzed. Ten random conidia from CP3002 consisted of five dual maters and five *Mat1-2*. Seven dual maters, two *Mat1-1* strains, and one *Mat1-2* strain were present among ten conidia isolated from CP3003. The more frequent isolation of dual-mater conidia from CP3002 and 3003 than from CP3000 and CP3001 described above suggests that these dual maters from strain 4091-5-8 may be relatively more stable than the dual maters from strain 4136-4-3. The low frequency of perithecia produced in self-matings from CP3002 and CP3003 suggests that dual maters of *M. grisea* may not be self-fertile. However, the answer to that question requires the isolation of stable strains that carry both mating-type idiomorphs.

Restriction maps of the mating-type idiomorphs: To subclone the mating-type genes, the cosmids pCMT1 and pCMT2 were digested with eight restriction enzymes and the digests by an individual restriction enzyme were transformed into 4091-5-8 and 4136-4-3, respectively, to test whether the enzyme digestion destroys the mating function. The digests were also subjected to Southern analysis using pMAT1 and pMAT2 as probes. Based on these analyses, the *Mat1-1* and *Mat1-2* genes were located within 3- and 4-kb *Kpn*I fragments, respectively. These fragments were sufficient for mating function. The sequences flanking these *Kpn*I fragments were

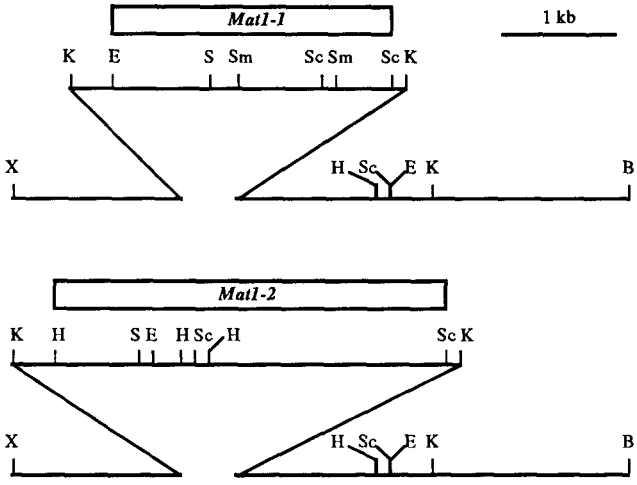

FIGURE 4.—Restriction maps of the mating-type idiomorphs and their flanking sequences. The idiomorphs are represented by boxes at the top of the maps. Restriction site abbreviations: B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; S, *Sal*I; Sc, *Sac*I; Sm, *Sma*I; X, *Xba*I.

cross-hybridized and all restriction sites are conserved (see Figure 4). Cross-hybridization with various restriction fragments within the *Kpn*I fragments revealed that the *Mat1-1* and *Mat1-2* genes are idiomorphs approximately 2.5 and 3.5 kb in length, respectively.

DISCUSSION

Using genomic subtraction, we have isolated the mating-type genes of *M. grisea*. As found in other fungal species, the genes are idiomorphs. Sizes of *M. grisea* idiomorphs (2.5 and 3.5 kb) are in between those of *N. crassa* (3.2 and 5.3 kb; GLASS *et al.* 1988) and *P. anserina* (3.8 and 4.7 kb; DEBUCHY and COPPIN 1992) and those of *C. heterostrophus* (1.2 and 1.3 kb; TURGEON *et al.* 1993). The genome sizes of both *N. crassa* and *M. grisea* are about 40 megabase pairs (HAMER *et al.* 1989; ORBACH *et al.* 1988). Our success in isolating DNA fragments corresponding to the mating-type genes shows that genomic subtraction is sensitive enough to isolate sequences corresponding to about 1/10,000th of the genome in these two fungal systems. With sensitivity of this magnitude, genomic subtraction can be a very powerful tool for cloning genes, if a method is available for generating deletion mutations in genes of interest. Several mutagens, such as high energy radiation (KELLEY *et al.* 1985; ZACHAR and BINGHAM 1982), diepoxyalkanes (ONG and DESERRES 1975; REARDON *et al.* 1987), chlorambucil (RUSSELL *et al.* 1989), and melphalan (RUSSELL *et al.* 1992), have been shown to cause deletion mutations at a high frequency.

We have demonstrated the feasibility of producing nearly isogenic strains of *M. grisea* by replacing the endogenous mating-type gene with the opposite mating-type gene. Using strain 4136-4-3, we derived nearly isogenic strains that mate with high fertility, yielding nearly 100% viable ascospores. However, our initial attempts to improve fertility in poorly fertile strains by gene replacement at the mating-type locus were unsuccessful. Nearly isogenic pairs produced from strain 4136-2-2 mated, but the viability of ascospores produced was low. This suggests that the poor viability of ascospores seen in the original crosses of this strain were not due to differences in chromosome arrangement between the mating partners. It is likely that this strain contains mutation(s) outside the mating-type locus that decrease ascospore viability. Numerous attempts to improve the fertility of the rice pathogen O-135 by replacement of its mating-type idiomorph with the *Mat1-2* idiomorph from a fully fertile laboratory strain also failed.

CP2738, a transformant of 4091-5-8 that was switched for mating type, produced numerous barren perithecia in crosses with 4091-5-8 and K76-79, but normal perithecia in crosses with the unrelated field isolates Guy11 and G-71. The possibility of a preexisting recessive mutation in a gene of 4091-5-8 that is necessary for ascospore development was ruled out because dual maters of 4091-5-8, CP3002 and CP3003, normally produced asci in self-crosses as well as in crosses with 4091-5-8. The other possibility is that a mutation occurred in the transgene during the transformation event. If so, the expression of the phenotype of this mutation must be dependent on genetic background. The similar phenotype in crosses with 4091-5-8 and K76-79 is probably due to a similar genetic background between 4091-5-8 and K76-79 (4091-5-8 is a progeny from a cross between K76-79 and WGG-FA40.). The size of the *Pst*I fragment corresponding to *Mat1-1* in CP2738 is slightly smaller than that of other *Mat1-1* strains (lane 12 of Figure 3), suggesting that the *Mat1-1* gene in CP2738 might have a small deletion. If the *Mat1-1* gene in CP2738 gained a mutation during the transformation event, characterization of this mutation will provide insight into the function of the mutated region of the *Mat1-1* gene. Recently, GLASS and LEE (1992) isolated mutants of *N. crassa* that show a similar phenotype to that of CP2738 by mutating a flanking region of *mtA-1* (an open reading frame that appears to confer fertility and heterokaryon incompatibility) in *MAT A*.

Dual maters of other Ascomycetes showed different levels of fertility depending on whether the endogenous mating-type gene or the transgene was required to function (GLASS *et al.* 1988; PICARD *et al.* 1991; TURGEON *et al.* 1993). Crosses in which the introduced gene functioned produced many perithecia that were devoid of asci, but crosses in which the endogenous mating-type gene functioned produced normal perithecia filled with asci. In *M. grisea* it is not clear whether the same is true because of rapid deletions of one mating-type gene during asexual growth of dual maters. We are currently attempting to construct dual maters that contain both

mating-type genes in a stable arrangement. Analysis of ascospores showed that dual maters of *P. anserina* occasionally lose the mating-type gene that is present in the tester (PICARD *et al.* 1991). However, it is not known when deletions of the mating-type gene occur. In *P. anserina* the transforming mating-type gene integrated in tandem to the endogenous gene in many transformants. It remains to be investigated whether the transforming mating-type gene actually integrated in tandem to the endogenous gene in dual maters that we examined and why some dual maters (*e.g.*, CP3000 and CP3001) appear to have higher deletion rates than others (*e.g.*, CP3002 and CP3003).

Early attempts to identify the mating type of *M. grisea* field isolates that infect rice by crossing to fertile tester isolates identified the mating type of only 52% of the isolates, because the remaining isolates did not mate with any of the testers (NOTTEGHEM and SILUÉ 1992; YAEGASHI and YAMADA 1986). The identification of mating types of field isolates in the future will be facilitated by using molecular probes instead of crossing to tester strains.

We are grateful to R. L. METZENBERG and J. GROTELUESCHEN for providing *N. crassa* strains and pOKE76. We would like to thank D. STRAUS and F. M. AUSUBEL for providing us a protocol for genomic subtraction. We also thank J. A. SWEIGARD for critical reading of the manuscript.

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Communicating editor: R. H. DAVIS