Enhanced Transformation in *Magnaporthe grisea* by Restriction Enzyme Mediated Integration of Plasmid DNA

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ABSTRACT

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To use insertional mutagenesis for gene tagging, we tested whether transformation of *Magnaporthe grisea* could be enhanced by restriction enzyme mediated integration (REMI) of plasmid DNA. The integrative plasmid pAN7-2 was linearized by restriction endonucleases *Hin*dIII, *Bam*HI, *Bgl*II, and *Eco*RV, and used to transform *M. grisea* strain Guy11 in the presence of the corresponding enzyme. Transformation frequency was increased by the incorporation of restriction enzymes, but the level of enhancement appeared to be enzyme-specific. An increase of up to 10-fold (75–200 transformants/μg DNA) was achieved with *Bam*HI and *Bgl*II, but only a two-fold increase was observed with *Hin*dIII. The optimal enzyme concentration for enhanced transformation also varied among enzymes (e.g., 20 units per milliliter for *Bam*HI vs. 200 units per milliliter for *Hin*dIII). Transformation efficiency appeared to be correlated with frequency of REMI. Southern blot analysis of the *Bam*HI-transformants showed that 72% of the plasmid integrations were at *Bam*HI sites whereas 28% of the integrations in *Hin*dIII-transformants were at *Hin*dIII sites. In *Eco*RV-mediated transformation, 42% of the integrations were at *Eco*RV sites. REMI transformation has allowed us to generate a large number of random insertions in the fungal genome from which mutant genes can be isolated.

Additional keyword: rice blast fungus.

Magnaporthe grisea (Hebert) Barr is an ascomycete that causes the blast disease of rice (10). Rapid progress has been made in the genetic and molecular analysis of the fungus (17); however, strategies to clone genes remain limited due to the lack of an efficient transformation system. Integrative transformation in *M. grisea* generally yields only 5–10 transformants per μg vector DNA (3,8,11). Thus far, most gene cloning experiments in *M. grisea* have been based upon chromosome walking (6,18). Map-based cloning, however, is time-consuming and often complicated by the presence of abundant repetitive sequences (4,16). An alternative to map-based cloning is insertional mutagenesis, in which genes are inactivated by the integration of a selectable marker. The sequences of the inactivated gene can then be recovered by isolation of the marker-containing genomic DNA fragment.

A prerequisite for insertional mutagenesis is an efficient transformation system from which a large number of random insertional mutants can be readily obtained. Schiestl and Petes (15) first reported a seven-fold increase in the transformation frequency in *Saccharomyces cerevisiae* by transforming yeast with a *Bam*HI-linearized heterologous DNA fragment in the presence of the enzyme *Bam*HI. More significantly, the heterologous DNA fragment with *Bam*HI-cohesive ends often integrated into *Bam*HI sites of the yeast genome. This phenomenon, called restriction enzyme mediated integration (REMI), was used to increase the transformation frequency in *Dictyostelium discoideum* and to generate random insertions in the genome (5). Although REMI mutagenesis has been recently reported in filamentous fungi (19,20), experimental details for the enhancement of transformation efficiency have not been available.

As a first step toward developing an efficient system for insertional mutagenesis in *M. grisea* in our laboratory, we evaluated the effects of different restriction enzymes and enzyme concentrations on transformation efficiency. We found that transformation efficiency in *M. grisea* was increased by up to 10-fold by REMI and that the level of enhancement correlated with the frequency of REMIs. Blunt-ended plasmid generated by *Eco*RV also appeared to direct a significant proportion of integrations to *Eco*RV sites of the host genome.

MATERIALS AND METHODS

Fungal strain, plasmid, and media. A hermaphroditic strain of *M. grisea,* Guy11, was used as the recipient host in all transformation experiments (7). The fungus was cultured routinely on oatmeal agar and stored on dry paper disks at −20 C (9). For the isolation of DNA, the fungus was grown in liquid complete medium (CM, 0.5% sucrose, 0.6% yeast extract, 0.6% casein hydrolysate) as described (2). The integrative plasmid pAN7-2 contains an *Escherichia coli* hygromycin B phosphotransferase gene linked to *Aspergillus nidulans* regulatory sequences (12). Circular pAN7-2 has been used to transform *M. grisea* at a frequency of approximately 5–10 transformants per μg DNA (8). The plasmid contains unique restriction sites for *Bam*HI, *Bgl*II, *Hin*dIII, and *Eco*RV.

Preparation of plasmid DNA. Plasmid pAN7-2 was isolated from *E. coli* strain DH5α by the alkaline extraction method and purified by cesium chloride gradient ultracentrifugation as described in Sambrook et al (13). The plasmid was linearized by digesting 1 μg of DNA with 10 units of the respective restriction enzyme in a 15-μl reaction for 1.5–2 h following supplier's instructions. In the initial experiments with *Hin*dIII and *Bam*HI, different methods were used to inactivate the residual enzymes before transformation. In experiment I, the enzymes were inactivated by incubation at 70 C for 15 min. In experiment II, the linearized plasmid was subjected to one cycle of phenol-chloroform extraction. In subsequent experiments with additional restriction enzymes (*Bgl*II and *Eco*RV), the linearized plasmid was extracted three times with phenol-chloroform.

REMI transformation. The transformation protocol was the same as described by Leung et al (8) except that 20% sucrose instead of 1.2 M sorbitol was used as the osmotic stabilizer. Briefly,

protoplasts were produced by digesting mycelial pellets in a digestion buffer (20% sucrose, 20 mM trisodium citrate, 50 mM EDTA, pH 8.0) containing 40 mg/ml Novozyme (Novo Biolab, Wilton, CT) for 2–3 h. Protoplasts were washed and maintained in STC buffer (20% sucrose, 25 mM Tris-HCl pH 7.5, 25 mM $CaCl_2$) until transformation.

Concentrations of *Hin*dIII and *Bam*HI ranging from 20 to 600 units per milliliter transformation mix were tested. Approximately 1 μg of the linearized DNA was mixed with different amounts of enzyme. To avoid damage to protoplasts, the enzyme was mixed with the plasmid DNA in STC before addition to the protoplast suspension. For each transformation treatment, 150 μl of STC containing 1 μg of linear DNA with or without enzyme was added to 100 μl of protoplast suspension (adjusted to $0.5–1.0 \times 10^8$ protoplasts per milliliter) in a 50-ml centrifuge tube. The same amount of circular plasmid DNA was used in the control transformation. After 20 min incubation on ice, 2 ml of polyethylene glycol 3350 (60% in 25 mM $CaCl_2$, 25 mM Tris-HCl, pH 7.5) was added in a dropwise manner. After chilling on ice for an additional 20 min, 30 ml of ice-cold STC was added gently to this mixture and protoplasts were recovered by centrifugation at 2,000 *g* for 12 min.

The protoplast mixture was resuspended in liquid regeneration medium (CM + 20% sucrose) and incubated without shaking at room temperature overnight. The suspension was mixed with molten regeneration agar (CM containing 20% sucrose and 2% agar maintained at 45 C) and poured into 10-cm-diameter petri plates. Solidified agar was overlaid with molten top agar (0.7%) containing 300 μg/ml of hygromycin B (maintained at 45–50 C). Plates were incubated at 26 C and the frequency of transformation was recorded by counting the primary transformants that emerged from the top-agar about 6–8 days after overlay. Transformation frequencies were confirmed by transferring primary transformants onto CM agar plates containing 250 μg/ml of hygromycin B. Selected hyphal-tip or single conidial cultures of the primary transformants were subjected to molecular analysis.

Southern blot analysis. For DNA extraction, mycelium of transformants was grown in CM broth without selection for hygromycin B resistance. Genomic DNA was isolated from lyophilized mycelium using the CTAB method as described (1). Enzyme-digested DNA was electrophoresed in 0.7% agarose, blotted onto Hybond N+ membrane (Amersham, Chicago, IL) using standard techniques (13). pAN7-2 was labeled with digoxigenin-dUTP (Boehringer Mannheim, Indianapolis, IN) and was used to probe genomic blots of transformants as described by the manufacturer's instructions. Hybridization signals were detected by chemiluminescence, using CSPD as substrate for alkaline phosphatase (Tropix Inc., Bedford, MA).

RESULTS

The effects of enzyme concentration on transformation frequency were tested using a range of concentrations of *Hin*dIII and *Bam*HI (Table 1). In the initial experiments, residual enzyme activity was removed from the linearized plasmid either by heat inactivation (experiment I) or by one cycle of phenol-chloroform extraction (experiment II). From these experiments, four significant observations were made. First, although the residual enzyme activity was presumably eliminated, transformation frequency was increased using linearized pAN7-2. Second, transformation frequencies declined at high concentrations of enzyme. At 600 units per milliliter of either *Hin*dIII or *Bam*HI, the transformation frequencies were less than those obtained with circular DNA. Third, transformation frequency was enhanced more with *Bam*HI than with *Hin*dIII at low concentrations of the enzyme; however, there was a rapid decline in transformation frequencies at high concentrations of *Bam*HI (200–600 units per milliliter). Finally, there was no observable decrease in the viability of protoplasts at the highest concentration of enzymes.

Additional experiments were conducted to determine whether enhancement in transformation frequency could be obtained with other enzymes (Table 2). Results from experiments I and II suggested that either the linear plasmid was highly recombinagenic or that there was residual enzyme activity after heat treatment or one cycle of phenol-chloroform extraction. To ensure that all the residual enzyme was removed, the linearized plasmid was subjected to three cycles of phenol-chloroform extraction. After this exhaustive extraction, the transformation frequency obtained by the linear plasmid was similar to that with the circular plasmid (e.g., *Bam*HI treatment in Table 2), supporting our hypothesis that the enhanced transformation obtained by linear plasmids in experiments I and II was due to residual enzyme activity. Consistent with results in experiments I and II shown in Table 1, an increase in transformation frequency was observed by using *Bam*HI and *Bgl*II in REMI transformation. However, when *Bam*HI-linearized plasmid was mixed with 20 or 80 units per milliliter of *Apa*I (which does not cut pAN7-2 and produces incompatible ends), enhanced transformation was not observed, suggesting that compatible ends were essential for enhancement. A blunt-end plasmid linearized with *Eco*RV yielded a slightly higher transformation frequency than plasmids linearized by either *Bam*HI or *Bgl*II. When 80 units per milliliter of *Eco*RV was added to the transformation mixture, a slight increase in transformation frequency was observed.

To determine the patterns of plasmid integration, DNA from transformants were digested with the respective enzymes used to generate the transformants, and subjected to Southern analysis using pAN7-2 as the hybridization probe. If integration is mediated by the compatible ends generated by the restriction enzyme, the two restriction recognition sites at the ends of the inserted plasmid would be restored after integration. Thus, hybridization of a plasmid-size fragment (9.2 kb) in transformant DNA digested with the same enzyme used in transformation provides evidence of a REMI event.

Four basic types of integration patterns were observed among the transformants analyzed. Type 1 transformants showed a single

TABLE 1. Number of transformants generated by different plasmid treatments in transformation of *Magnaporthe grisea*

Enzyme	Experiment[a]	Circular plasmid	Linearized plasmid	Linearized plasmid plus enzyme (unit/ml)				
				40	80	200	400	600
*Hin*dIII	I	30[b]	37	ND[c]	38	54	21	13
	II	33	67	39	79	94	70	22
*Bam*HI	I	15	169	ND	65	ND	21	ND
	II	34	234	169	113	31	4	6

[a] In both experiments, the plasmid pAN7-2 was linearized using 10 units of the respective restriction enzymes. In experiment I, the restriction enzymes were heat treated at 70 C for 15 min. In experiment II, the restriction enzymes were removed by one cycle of phenol-chloroform extraction.
[b] Number of transformants per μg pAN7-1 DNA.
[c] Not determined.

TABLE 2. Enhancement of transformation frequency in *Magnaporthe grisea* by different plasmid-enzyme combinations

Plasmid treatment[a]	Enzyme added	Experiment	Units of enzyme/ml transformation mixture		
			0	20	80
*Bam*HI-linearized	*Bam*HI	1	12[b]	ND[c]	78
	*Bam*HI	2	20	56	34
	*Apa*I	1	ND	4	6
*Bgl*II-linearized	*Bgl*II	1	12	30	135
	*Bgl*II	2	12	56	72
*Eco*RV-linearized	*Eco*RV	1	36	42	66
	*Eco*RV	2	28	26	48

[a] After linearization, the plasmid was extracted with phenol-chloroform three times.
[b] Number of transformants per μg pAN7-2 DNA.
[c] Not determined.

9.2-kb band that indicated that both restriction sites were restored, suggesting a REMI event (Fig. 1B, lanes 2 and 3; Fig. 1C, lanes 1, 3, and 4). A REMI event with tandem integration could also occur as indicated by an intense 9.2-kb hybridization band (Fig. 1A, lane 6). Type 2 transformants showed a single band larger than 9.2 kb (Fig. 1A, lane 1; Fig. 1B, lane 1). This indicates an integration that did not restore either one or both restriction sites, or an illegitimate integration of the linear plasmid. Type 3 transformants showed a single 9.2-kb band and a band larger than 9.2 kb, suggesting a combination of integration events as occurred in Type 1 and Type 2 transformants (Fig. 1A, lane 3). Type 4 transformants showed a 9.2-kb band plus other bands of varying sizes (Fig. 1A, lane 2). These patterns could result from REMI events, tandem integrations, or a combination of both.

To determine whether the integration sites by REMI transformation were random, transformant DNA was digested with *Eco*RI and probed with pAN7-2. Since pAN7-2 produces two fragments (6.8 kb and 2.4 kb) when digested with *Eco*RI, the copy number and pattern of integrations could be inferred based on these two diagnostic bands. Figure 2 shows the hybridization patterns of the same transformant DNA as Fig. 1, but digested with *Eco*RI. Each transformant had a unique hybridization pattern, indicating that plasmid integrations were at different sites in the genome. Of 43 transformants analyzed by *Eco*RI, 60% were the result of single-copy integrations as shown by a typical pattern of the 2.4-kb fragment plus two hybrid fragments containing flanking sequences from the fungal genome (Fig. 2B, lanes 2 and 5). Five percent were tandem integrations (detection of both the 2.4- and 6.8-kb bands as illustrated in Fig. 2A, lane 2) and 35% were integrations at more than one site (Fig. 2A, lane 4; Fig. 2B, lane 4). There was no apparent difference in the number of integrations between transformants obtained from low (100 units per milliliter) and high (600 units per milliliter) concentrations of *Bam*HI (data not shown).

Table 3 summarizes the number of REMI events in transformation experiments using different restriction enzymes. By counting Type 1 and Type 3 transformants, we obtained a conservative estimate of REMI events. Of the three enzymes tested, the highest number of REMI events (72%, 13 out of 18) was found with *Bam*HI-mediated transformation. With *Hin*dIII-mediated transformation, 28% (8 out 28) transformants were REMI events. Despite the fact that *Eco*RV generates blunt ends, 42% of the transformants generated by *Eco*RV-mediated transformation produced a 9.2-kb plasmid-size fragment, indicating that a significant proportion of integrations were at *Eco*RV sites of the

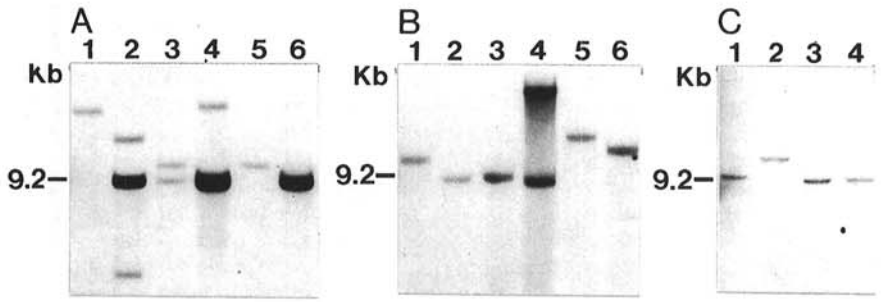

Fig. 1. Southern analyses of transformants derived from restriction enzyme mediated transformation. Transformant DNA was digested with the respective enzymes and probed with pAN7-2. **A,** *Bam*HI-mediated transformants. DNA digested with *Bam*HI. **B,** *Hin*dIII-mediated transformants. DNA digested with *Hin*dIII. **C,** *Eco*RV-mediated transformants. DNA digested with *Eco*RV. Approximately 3 μg of digested DNA was loaded in each lane, electrophoresed in 0.7% agarose, blotted, and probed with pAN7-2. A single 9.2-kb hybridization band indicates a REMI event (e.g., lanes 2 and 3 in **B,** lanes 1, 3, and 4 in **C**). Other types of transformants are explained in text.

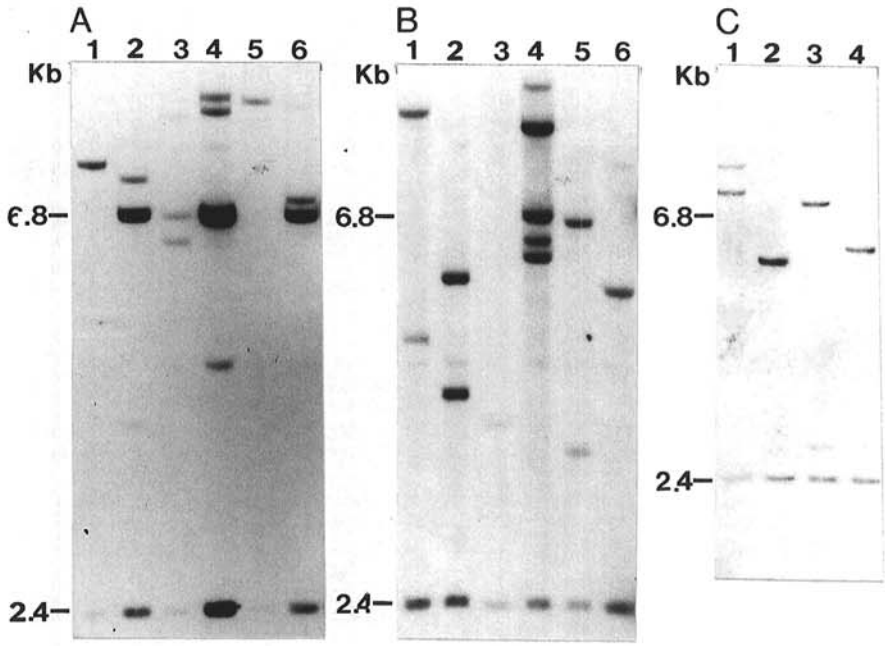

Fig. 2. Southern analyses of transformants derived from restriction enzyme mediated transformation. Transformant DNAs corresponding to those in Fig. 1 were digested with *Eco*RI and probed with pAN7-2. **A,** *Bam*HI-mediated transformants. **B,** *Hin*dIII-mediated transformants. **C,** *Eco*RV-mediated transformants. All integrations are different, indicating random insertions in the independent transformants. Restoration of the 2.4- and 6.8-kb bands indicates a tandem integration event (e.g., lanes 2 and 6 in **A,** lanes 4 and 5 in **B**).

fungal genome (Fig. 1C). When only linearized plasmids were used in transformation, the majority of the transformants (85%) showed a single fragment larger than 9.2 kb, suggesting that the plasmid integrated into the genome in the linear form but one or both of the restriction sites were not restored. However, in four cases, REMI events were observed among transformants obtained with plasmids linearized by *Bam*HI and *Eco*RV (Table 3).

DISCUSSION

We have demonstrated that incorporation of restriction enzymes in transformation can increase the transformation efficiency in *M. grisea*. The level of enhancement, however, varies among enzymes. Incorporation of a relatively small amount of *Bam*HI or *Bgl*II (20–80 units per milliliter) enhanced transformation efficiency by up to 10-fold. This is in contrast to the high concentrations of enzymes used in the studies of yeast and slime mold (5,15), and may explain the lack of enhancement in transformation efficiency observed by other researchers (R. Dean, *personal communication*). The optimal concentration for enhancement may be less than 20 units per milliliter for *Bam*HI, since carry-over enzyme activity after digestion of the plasmid is sufficient to enhance transformation. In contrast, only a two-fold increase in transformation frequency was achieved with *Hin*dIII at a high enzyme concentration (200 units per milliliter). Schiestl et al (14) showed that REMI in yeast was effective with *Bam*HI but not with *Eco*RI. They suggested that *Eco*RI may not enter the cell or does not function properly once inside the cell. On the other hand, Kuspa and Loomis (5) found that both *Eco*RI and *Bam*HI are effective in enhancing transformation in *D. discoideum*. In *M. grisea,* both *Bam*HI and *Hin*dIII appeared to be able to enter the cell as evidenced by the decline in transformation frequency at high concentrations of enzymes (Table 1). The results from yeast and *Dictyostelium,* together with ours, suggest that the activity of a particular restriction enzyme in mediating transformation is dependent upon the recipient host.

According to Schiestl and Petes (15), REMI is a result of the cleavage of chromosomal DNA by the introduced enzyme, followed by pairing of the cohesive ends between the linear plasmid and the cleaved chromosomal DNA. The hybrid junctions are then ligated and the restriction recognition sites at both ends of the inserted plasmid are restored. REMI events are shown in *M. grisea* by digesting genomic DNA from transformants with the same restriction enzyme used for linearization of plasmid DNA and detection of the intact plasmid in Southern analysis. Increased transformation frequency was correlated with the frequency of REMI events. Results of the experiment using *Bam*HI-linearized plasmid and an incompatible enzyme *Apa*I further indicate that compatible cohesive ends are essential for enhanced transformation. The four cases of REMI events observed in using plasmids linearized by *Bam*HI and *Eco*RV (Table 3) were most likely caused by residual enzyme activity. We observed a slight increase in transformation frequency when *Eco*RV was used to mediate integration. This could be explained by the generation of blunt-end cuts in the genome that facilitate integration. Further elucidation of the blunt-end mediated integration will require experimentation with various blunt-end producing enzymes as well as sequence analyses of the integration junctions.

Since Schiestl and Petes (15) first reported REMI in yeast, REMI has been successfully used in *D. discoideum* as a tool to generate insertional mutations (5). In *D. discoideum,* about 0.25% of the REMI transformants were developmental mutants (5). Once the gene is tagged, sequence of the inactivated gene can be retrieved by digesting the mutant DNA with an enzyme that cuts outside the integrated plasmid, followed by re-circularization and transformation into *E. coli* under ampicillin selection. With REMI mutagenesis, the recovery of genomic sequences flanking the inserted plasmid can be greatly facilitated because the tagged sequence is flanked by known restriction sites (the REMI integration sites and the restriction sites used to retrieve the plasmid). The enhanced efficiency achieved by REMI transformation has made it feasible to generate over a thousand integrative transformants in *M. grisea* in a few experiments. Thus far, we have recovered five mutants that exhibit defects in sporulation (two), auxotrophy (one), and reduced pathogenicity (two), from about 600 REMI transformants examined. All the mutant phenotypes have been shown to cosegregate perfectly with hygromycin B resistance in ascospore progeny (Z. Shi, Y. Shi, and H. Leung, *unpublished data*). This frequency (0.8%) is similar to that obtained in *Dictyostelium* (5).

An important consideration for effective insertional mutagenesis is whether the insertional events are randomly distributed in the genome. The integrations observed in the *M. grisea* transformants analyzed thus far appear to be random, which is consistent with the results from yeast and *Dictyostelium.* An advantage of REMI mutagenesis is that the target insertion sites can be changed simply by using enzymes with different recognition sites. We have found that *Bam*HI and *Bgl*II are equally effective in enhancing transformation in *M. grisea.* A full coverage of the genome can be ensured by using *Bam*HI and *Bgl*II or other effective enzymes to generate the mutant collection.

TABLE 3. Number of restriction enzyme mediated integration (REMI) events in *Magnaporthe grisea* transformants using different plasmid treatments

Plasmid treatment in transformation experiment	No. of transformants analyzed	Nature of integration	
		REMI[a]	non-REMI
*Bam*HI-linearized	8	2	6
*Bam*HI-linearized + *Bam*HI	18	13	5
*Hin*dIII-linearized	10	0	10
*Hin*dIII-linearized + *Hin*dIII	28	8	20
*Eco*RV-linearized	10	2	8
*Eco*RV-linearized + *Eco*RV	19	8	11

[a] Detection of a 9.2-kb plasmid band when transformant DNA was digested with the same enzyme used in transformation. Only type 1 and type 3 patterns, as explained in the text, are considered REMI events.

LITERATURE CITED

1. Borromeo, E. S., Nelson, R. J., Bonman, J. M., and Leung, H. 1993. Genetic differentiation among isolates of *Pyricularia* infecting rice and weed hosts. Phytopathology 83:393-399.
2. Crawford, M. S., Chumley, F. G., Weaver, C. G., and Valent, B. 1986. Characterization of the heterokaryotic and vegetative diploid phases of *Magnaporthe grisea.* Genetics 114:1111-1129.
3. Daboussi, M. J., Djeballi, A., Gerlinger, C., Blaiseau, P. L., Bouvier, I., Cassan, M., Lebrun, M. H., Parisot, D., and Brygoo, Y. 1989. Transformation of seven species of filamentous fungi using the nitrate reductase gene of *Aspergillus nidulans.* Curr. Genet. 15:453-456.
4. Hamer, J. E., Farrall, L., Orbach, M., Valent, B., and Chumley, F. G. 1989. Host species-specific conservation of a family of repeated DNA sequences in the genome of a fungal plant pathogen. Proc. Natl. Acad. Sci. USA 86:9981-9985.
5. Kuspa, A., and Loomis, W. F. 1992. Tagging developmental genes in *Dictyostelium* by restriction enzyme-mediated integration of plasmid DNA. Proc. Natl. Acad. Sci. USA 89:8803-8807
6. Leong, S. A., Farman, M., Smith, J., Budde, A., Tosa, Y., and Nitta, N. 1994. A molecular-genetic approach to the study of cultivar specificity in the rice blast fungus. Pages 87-110 in: Rice Blast Disease. R. S. Zeigler, S. A. Leong, and P. S. Teng, eds. CAB International Press, Wallingford, Oxon, UK.
7. Leung, H., Borromeo, E. S., Bernardo, M. A., and Notteghem, J. L. 1988. Genetic analysis of virulence in the rice blast fungus *Magnaporthe grisea.* Phytopathology 78:1227-1233.
8. Leung, H., Lehtinen, U., Karjalainen, R., Skinner, D., Tooley, P., Leong, S., and Ellingboe, A. 1990. Transformation of the rice blast fungus, *Magnaporthe grisea,* to hygromycin B resistance. Curr. Genet. 17:409-411.
9. Leung, H., and Taga, M. 1988. *Magnaporthe grisea* (*Pyricularia* species), the blast fungus. Adv. Plant Path. 6: 175-188.
10. Ou, S. H. 1985. Rice Diseases. Commonwealth Mycological Institute. Kew, Surrey, England.
11. Parsons, K. A., Chumley, F. G., and Valent, B. 1987. Genetic transformation of the fungal pathogen responsible for rice blast disease. Proc. Natl. Acad. Sci. USA 84:4161-4165.

12. Punt, P. J., Oliver, R. P., Dingemanse, M. A., Pouwels, P. H., and van den Hondel, C. A. M. J. J. 1987. Transformation of *Aspergillus* based on the hygromycin B resistance marker from *Escherichia coli*. Gene 56:117-124.
13. Sambrook, J., Fritsch, E. F., and Maniatis, T. 1989. Molecular Cloning - A Laboratory Manual, 2nd ed. Cold Spring Harbor Laboratory Press. Cold Spring Harbor, NY.
14. Schiestl, R. H., Dominska, M., and Petes, T. D. 1993. Transformation of *Saccharomyces cerevisiae* with nonhomologous DNA: Illegitimate integration of transforming DNA into yeast chromosome and in vivo ligation of transforming DNA to mitochondrial DNA sequences. Mol. Cell. Biol. 13:2697-2705.
15. Schiestl, R. H., and Petes, T. D. 1991. Integration of DNA fragments by illegitimate recombination in *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. USA 88:7585-7589.
16. Skinner, D. Z., Budde, A. D., Farman, M. L., Smith, J. R., Leung, H., and Leong, S. A. 1993. Genome organization of *Magnaporthe grisea:* Genetic map, electrophoretic karyotype, and occurrence of repeated DNAs. Theor. Appl. Genet. 87:545-557.
17. Valent, B., and Chumley, F. G. 1991. Molecular genetic analysis of the rice blast fungus, *Magnaporthe grisea*. Annu. Rev. Phytopath. 29:443-467.
18. Valent, B., and Chumley, F. G. 1994. Avirulence genes and mechanisms of genetic instability in the rice blast fungus. Pages 111-134 in: Rice Blast Disease. R. S. Zeigler, S. A. Leong, and P. S. Teng, eds. CAB International Press, Wallingford, Oxon, UK.
19. Valent, B., Sweigard, J. A., Orbach, M. J., Kang, S., Farrall, L., Carroll, A. M., and Chumley, F. G. 1994. Molecular characterization of genes pathogenicity and host specificity in the rice blast fungus. Page 228 in: Prog. Abstr. Int. Mycol. Cong., 5th.
20. Yang, G., Turgeon, B. G., and Yoder, O. C. 1994. Molecular analysis of toxin production by *Cochliobolus heterostrophus* using tagged mutations and heterokaryons. Page 249 in: Prog. Abstr. Int. Mycol. Cong., 5th.