***Magnaporthe grisea* Pathogenicity Genes Obtained Through Insertional Mutagenesis**

James A. Sweigard,¹ Anne M. Carroll,¹ Leonard Farrall,² Forrest G. Chumley,³ and Barbara Valent²

¹Central Research and Development Department, and ²DuPont Agricultural Products, E. I. du Pont de Nemours and Co., Inc., P.O. Box 80402, Wilmington, DE 19880-0402 U.S.A.; and ³DuPont Agricultural Products, Barley Mill Plaza, P.O. Box 80037 Wilmington, DE 19880-0037 U.S.A.

Accepted 1 February 1998.

We have initiated a mutational analysis of pathogenicity in the rice blast fungus, *Magnaporthe grisea*, in which hygromycin-resistant transformants, most generated by restriction enzyme-mediated integration (REMI), were screened for the ability to infect plants. A rapid primary infection assay facilitated screening of 5,538 transformants. Twenty-seven mutants were obtained that showed a reproducible pathogenicity defect, and 18 of these contained mutations that cosegregated with the hygromycin resistance marker. Analysis of eight mutants has resulted in the cloning of seven *PTH* genes that play a role in pathogenicity on barley, weeping lovegrass, and rice. Two independent mutants identified the same gene, *PTH2*, suggesting nonrandom insertion of the transforming DNA. These first 7 cloned *PTH* genes are described.

The parasitic interaction between pathogenic fungi and their plant hosts is a complex biological process. For example, for *Magnaporthe grisea*, the causal agent of blast on many different grasses, the pathogen cycle involves (minimally) the following developmental sequence: deposition of a conidium on the plant surface, germination of the conidium to form a germ tube, differentiation of the germ tube into a specialized infection structure called an appressorium, penetration of the plant leaf surface by the melanized appressorium via a penetration peg, differentiation of the penetration peg into a secondary hypha that then grows throughout the invaded plant cell, escape from the first infected plant cell and growth throughout the surrounding plant tissue, production of aerial conidiophores, production of conidia, and finally release of the conidia to complete the cycle. In this developmental sequence, only the melanization and pressurization of the appressorium are understood at a detailed level (Howard and Valent 1996). Moreover, the entire interaction is apparently modified by preformed and active plant defenses. To begin to understand the mechanisms of pathogenesis we have conducted a screen for mutants of *M. grisea* with altered pathogenicity.

The requirements for a successful mutant hunt are daunting for a filamentous fungal pathogen. Ideally the following would need to be in place to conduct a mutant hunt in a fungal pathogen: a random mutagenesis system, a rapid infection assay conducive to large numbers of assays, a sexual cycle to establish the genetics of the mutation, and an efficient transformation system for subsequent gene cloning. All of these requirements for a screen for nonpathogenic mutants are in place for *M. grisea*. The advantages of this fungus as a molecular genetic organism for the investigation of fungal pathogen/plant interactions have been documented (Valent and Chumley 1991). Nevertheless, cloning of any genes by complementation would, individually, still represent a major undertaking. We therefore decided to explore insertional mutagenesis via transformation to tag and thereby facilitate cloning of genes identified as playing a role in pathogenesis. We were initially encouraged to attempt insertional mutagenesis via integrative transformation because it had been successfully employed in *Aspergillus nidulans* (Diallinas and Scazzocchio 1989; Tilburn et al. 1990) and *Neurospora crassa* (Kang and Metzenberg 1993). Subsequently, methods for insertional mutagenesis were extended to include restriction enzyme-mediated integration (REMI). Initially discovered in *Saccharomyces cerevisiae* (Schiestl and Petes 1991), this technique has been extensively used in *Dictyostelium discoideum* (Kuspa and Loomis 1992). Among plant pathogens, REMI has been used to tag the *Tox1* locus in *Cochliobolus heterostrophus* (Lu et al. 1994), conidiation genes in *M. grisea* (Shi and Leung 1995), and genes required for pathogenicity in *Ustilago maydis* (Bölker et al. 1995). Here we report the tagging and cloning of genes involved in pathogenicity in *M. grisea* by both normal and REMI transformation. A preliminary review of REMI work in filamentous fungi has been published (Sweigard 1996).

RESULTS

Mutant hunt summary.

The search for mutants with altered pathogenicity was conducted with a variety of strains, vectors, transformation conditions, and assays (Tables 1 and 2). The vast majority of the transformants were generated in strain 4091-5-8 by REMI and were tested in a rapid infection assay on barley. From the total of 5,538 transformants examined, 27 were obtained that gave a consistent disease phenotype that segregated in crosses (Table 3). In 18 of these 27 mutants, the disease phenotype co-

Corresponding author: James A. Sweigard; Telephone: 1-302- 695-2523; Fax: 1-302- 695-8480; E-mail: Jim.Sweigard@usa.dupont.com

Nucleotide and/or amino acid sequence data have been submitted to GenBank as accession nos. AF027979, AF027980, AF027981, AF027982, and AF027983.

Table 1. Cosmids and plasmids

Cosmid or plasmid	Description or important characteristics ^a
pCB725	Hygromycin and ampicillin resistance
pCB819	Hygromycin and chloramphenicol resistance
pBARKS1	Bialaphos and ampicillin resistance
A8A2	Cosmid from 4392-1-6 library that complements <i>pth1</i>
A1F3	Cosmid from 4392-1-6 library that complements <i>pth2</i>
A26C8	Cosmid from 4392-1-6 library that complements <i>pth3</i>
A42F1	Cosmid from 4392-1-6 library that complements <i>pth4</i>
pCB821	Plasmid rescued from <i>HindIII</i> -cut <i>pth1</i> DNA
pCB880	Plasmid rescued from <i>HindIII</i> -cut <i>pth2</i> DNA
pCB889	pBC SK ⁺ + 6-kb <i>XhoI</i> fragment from A8A2; complements <i>pth1</i>
pCB890	Plasmid rescued from <i>HindIII</i> -cut <i>pth3</i> DNA
pCB902	Plasmid rescued from <i>Sall</i> -cut <i>pth4</i> DNA
pCB903	pBSII KS ⁺ + 2-kb <i>HindIII/NotI</i> fragment of A26C8; complements <i>pth3</i>
pCB914	pBSII KS ⁺ + 2.8-kb <i>SstII/EcoRI</i> fragment from A1F3; complements <i>pth2</i>
pCB924	pBSII KS ⁺ + 6-kb <i>SstI</i> fragment A42F1; complements <i>pth4</i>
pCB943	pBSII KS ⁺ Δ <i>SalI/BamHI</i>
pCB945	pUC19 + 4.4-kb <i>XbaI</i> fragment of A42F1; complements <i>pth4</i>
pCB946	pUC19 + 6-kb <i>HindIII</i> fragment of A1F3; complements <i>pth2</i>
pCB947	pCB943 + 6-kb <i>XhoI</i> fragment from pCB889
pCB948	pUC19 + 7-kb <i>BamHI</i> fragment of A26C8; contains <i>PTH3</i> and basis of <i>pth3::Hyg</i> gene disruption vector
pCB951	pCB947 Δ <i>SalI/BamHI</i> + 1.4-kb <i>SalI/BamHI</i> fragment of pCSN43 containing the <i>Hyg</i> gene; <i>pth1::Hyg</i>
pCB952	pSL1180 <i>XhoI</i> + 2.4-kb <i>SalI</i> fragment of pCSN43
pCB954	pCB948 <i>EagI</i> + pCSN43 <i>SalI/BamHI</i> (both pieces treated with Klenow); <i>pth3::Hyg</i> gene disruption vector
pCB965	pCB946 <i>EagI/NcoI</i> + pCB952 <i>NotI/NcoI</i> (partial); <i>pth2::Hyg</i> gene disruption vector
pCB985	pBARKS1 <i>XhoI/MluI</i> + pCB945 <i>SalI/MluI</i> fragment; complements <i>pth4</i>
pCB1029	3.5-kb <i>ApaI/KpnI</i> (partial) of genomic DNA from 4091-5-8; contains sulfonyleurea resistant form of acetolactate synthase
pCB1030	pCB945 <i>EagI/EcoRV</i> + pCB952 <i>NotI/XbaI</i> (Klenow filled-in) (<i>Xba</i> site destroyed in construction); <i>pth4::Hyg</i> gene disruption vector
pCB1110	cDNA corresponding to <i>PTH3</i>
pCB1199	Plasmid from λBarfix library complementing <i>pth8</i>
pCB1210	cDNA corresponding to <i>PTH2</i>
pCB1219	cDNA corresponding to <i>PTH8</i>
pCB1223	Plasmid from λBARFIX library complementing <i>pth10</i>
pCB1238	cDNA corresponding to <i>PTH10</i>
pCB1254	pCB1029 <i>XbaI/ApaI</i> + 3 Kb <i>XbaI/ApaI</i> (partial); complements <i>pth8</i>
pCB1273	pCB1265 <i>EcoRI/XhoI</i> + 5 Kb <i>EcoRI/XhoI</i> fragment complementing <i>pth10</i>
pCB1294	cDNA corresponding to <i>PTH1</i>
pCB1362	cDNA corresponding to <i>PTH9</i>
pCB1466	Plasmid rescued from <i>BclI</i> -cut <i>pth2-2</i> DNA
pCB1467	Plasmid rescued from <i>XhoI</i> -cut <i>pth8</i> DNA
pCB1468	Plasmid rescued from <i>XhoI</i> -cut <i>pth9</i> DNA
pCB1469	Plasmid rescued from <i>XhoI</i> -cut <i>pth10</i> DNA
pCB1470	Plasmid rescued from <i>BamHI</i> -cut <i>pth8</i> DNA
pCB1503	Plasmid from λBARFIX library complementing <i>pth9</i>

^a pCB725 and pCB819 from Sweigard et al. (1995) and pBARKS1 from Pall and Brunelli (1993). All other plasmids and cosmids constructed for this paper.

segregated with the hygromycin resistance marker and these mutations were considered “tagged” (i.e., an insertion directly in the gene or tightly linked to the gene). The disease phenotypes for some of the mutants are shown in Figure 1. We used genetic analysis to eliminate mutations that were clearly untagged and did not investigate rare nonparental progeny (e.g., *pth8*). The mutated genes were named *PTH* for pathogenicity and numbered consecutively to maintain consistency with the gene naming system for filamentous fungal pathogens (Yoder et al. 1986). A range of phenotypes was found including no symptoms, reduced numbers of normal lesions (types 3 to 5), reduced numbers of type 1 and 2 lesions, and normal numbers of all type 1 lesions (Table 3) (Valent et al. 1991). Transfor-

Table 2. Mutant hunt summary

Experiment	Strain	Plasmid	REMI ?	Assay	Tested (no.)	Mutants PTH
1	CP987	pCB725	No	Standard	176	0
2	4091-5-8	pCB725	No	Standard	432	1
3	4091-5-8	pCB819	No	Standard	140	0
4	4091-5-8	pCB819	Yes	Rapid	889	2–7
5	4091-5-8	pCB1004	Yes	Rapid	3901	8–25

Table 3. Segregation data

Name ^a	PTH ^b	Disease ^c	Vector ^d	Enzyme ^e	R ⁺ ^f	R [–]	S ⁺	S [–]
CP1427	1	B	pCB725	ccc ^g	0	9	6	0
I93	2-1	A	pCB819	<i>XhoI</i>	0	11	22	1
B23	3	D	pCB819	<i>HindIII</i>	1	14	30	0
D24	4	C	pCB819	<i>HindIII</i>	0	21	24	0
Bgl15	5	B	pCB819	<i>BglII</i>	0	13	21	0
Bgl13 ^h	6	B	pCB819	<i>BglII</i>	2	3	6	4
B83 ^h	7	A	pCB819	<i>HindIII</i>	5	2	10	7
PL3	8	C	pCB1004	<i>PstI</i>	1	6	16	1
E508	9	C	pCB1004	<i>EcoRI</i>	0	8	11	0
G2-467	10	E	pCB1004	<i>BglII</i>	0	10	11	0
K261	11-1	B	pCB1004	<i>KpnI</i>	0	7	15	0
K364	11-2	B	pCB1004	<i>KpnI</i>	0	4	12	0
Sma47	12	A	pCB1004	<i>SmaI</i>	0	26	22	0
Sma37	13	C	pCB1004	<i>SmaI</i>	0	16	11	0
A108 ^h	14	C	pCB725	ccc	7	6	3	3
A74 ^h	15	D	pCB1004	<i>ApaI</i>	2	14	22	8
Bcl641 ⁱ	2-2	A	pCB1004	<i>BclI</i>				
Bcl578	16	D	pCB1004	<i>BclI</i>	0	19	28	0
Bcl755	17	C	pCB1004	<i>BclI</i>	0	14	10	0
E193 ^h	18	D	pCB1004	<i>EcoRI</i>	2	21	17	8
G2-36	19	B	pCB1004	<i>BglII</i>	2	19	3	0
K384	20	D	pCB1004	<i>KpnI</i>	0	5	4	0
K386	21	C	pCB1004	<i>KpnI</i>	1	8	14	1
Eag74 ^h	22	B	pCB1004	<i>EagI</i>	14	6	12	5
Sal19 ^h	23	B	pCB1004	<i>SalI</i>	6	11	11	6
P103 ^h	24	C	pCB1004	<i>PstI</i>	3	2	5	5
T1-106 ^h	25	A	pCB1004	<i>SstI</i>	1	7	10	6

^a Our original designation.

^b Pathogenicity gene name.

^c Disease phenotype: A = No symptoms, B = Rare lesions, C = Significantly reduced numbers of mainly type 1 and 2 lesions, D = Wild-type or near wild-type numbers of type 1 and type 2 lesions. E = Nearly fully pathogenic, greatly reduced conidiation in culture. Lesion type described by Valent et al. (1991).

^d Vector used in transformation.

^e Restriction enzyme used in transformation.

^f Segregation data where R and S mean resistant and sensitive to hygromycin and + and - mean wild-type and mutant phenotype.

^g Covalently closed circular.

^h Segregation data suggests that these mutations were not tagged by the plasmid integration event.

ⁱ Segregation not determined.

Fig. 1. Leaves from infection assays. Barley leaves (cv. Bonanza) 6 days after inoculation. **A**, Wild-type 4091-5-8; **B**, *pth2-1*; **C**, *pth3*; **D**, *pth8*; **E**, *pth9*; **F**, *pth10*. To emphasize the reduced symptoms produced by the mutants, plants infected with the parental strain, 4091-5-8, were inoculated with 2×10^5 conidia, one-fifth the number of conidia used for mutant inoculation.

mutants were excluded from consideration if the disease phenotype varied considerably between independent monoconidial isolates in repeated assays, or if the disease phenotype failed to segregate as a single gene, or if the transformant crossed poorly.

General method of cloning and identification of tagged genes.

Wild-type genes corresponding to some of the mutant phenotypes have been cloned (Table 4). This was accomplished by obtaining the genomic DNA flanking the insertion point by plasmid rescue (see Materials and Methods). This rescued plasmid was then used to probe a Southern blot with DNA from the mutant and wild type to verify that the plasmid did indeed identify a plasmid integration event and was not a spurious, contaminating plasmid. This analysis also showed that in almost all cases a single integration event had occurred (data not shown). The rescued plasmid was then sequenced and/or used as a hybridization probe to screen *M. grisea* genomic libraries to identify wild-type plasmids or cosmids. These plasmids containing wild-type genomic DNA were tested for complementation of the pathogenicity defect in the mutant. The complementing DNA was delimited by subcloning, followed by complementation of the mutant to full pathogenicity. Table 4 lists the initial complementing plasmid and the smallest complementing plasmid for each mutant. In most cases, a cDNA has also been identified (Table 4). Gene location and identification have also been confirmed by gene disruption (Fig. 2) and/or introduction of frameshift mutations into the complementing plasmid by restriction site modification (restriction digestion followed by treatment with Klenow fragment of DNA polymerase and plasmid recircularization). The sequences of these genes have been deposited in GenBank (accession numbers listed in Table 4). Sequence similarities were determined with the GAP program (Devereaux et al. 1984). A specific discussion of each of the cloned genes is given below.

Specific mutants.

pth1. Sequence from the complementing region of plasmid pCB889 yielded a long open reading frame (ORF) with homology to *GRR1* from *Saccharomyces cerevisiae* (Flick and Johnston 1991). *GRR1* was initially isolated in yeast in a screen for mutants with an altered response to glucose repression (Bailey and Woodward 1984). Subsequent work, including cDNA sequencing, has confirmed that *PTH1* is a *GRR1* homolog (J. Beckermann, J. Sweigard, and D. Ebbole, *manuscript in preparation*).

pth2-1 and *pth2-2*. Genomic and cDNA sequencing of clones related to *PTH2-1* showed an ORF with homology to numerous acyltransferases, including the choline acetyltransferase of mouse and carnitine palmitoyl transferase of rat. The strongest homology (41% identity, 52% similarity; Fig. 3) was to carnitine acetyl transferase of *S. cerevisiae* (GenBank accession no. P32796). The DNA sequence at the insertion site of *pth2-2* (see Figure 2) was identical to sequence found in *PTH2-1*. Since the sequence from the insertion site in *pth2-2* is from a different region of the gene than the *pth2-1* insertion and since these mutants were obtained in separate experiments, they are clearly independent mutants. The *pth2-2* mutant was also complemented by the same plasmid that complements *pth2-1*, pCB914.

pth3. The *pth3* mutant showed a partial requirement for histidine, growing at about 40% of the rate of wild type in radial growth assays on minimal medium (Fig. 4). Sequence of clones that complemented *pth3* showed strong homology to imidazole glycerol phosphate dehydratase (IGPD), the sixth step in histidine biosynthesis (63% amino acid identity, 72% amino acid similarity with the *S. cerevisiae* protein). The leaky nature of the histidine requirement is most likely due to the location of the insertion in the 5' region of the gene, 240 bp from the translation initiation codon (Fig. 2).

pth4. Sequence from the insertion site of the *pth4* mutant showed that the transforming plasmid had integrated into *CPKA*, a *M. grisea* gene encoding a cyclic AMP (cAMP)-dependent protein kinase homolog (Mitchell and Dean 1995). One end of the insertion occurred 8 amino acids from the end of the protein. The sequence of the other insertion junction was not determined. Since the insertion in this gene was so near the end of the gene it is possible that the insertion actually affected a gene downstream from *CPKA* and not *CPKA* itself. However, *pth4* can be complemented with a plasmid containing only *CPKA* (on a 2.5-kb insert; Fig. 2), indicating that the insertion actually affects *CPKA*. The *pth4* mutant has been further characterized by Xu et al. (1997).

pth8. Sequence from both sides of the insertion site in the *pth8* mutant yielded ORFs with strong homology to the middle of a hypothetical yeast protein of 1,198 amino acids (GenBank accession no. S51434). On one side of the insertion (pCB1467) there is an ORF with 66% identity over 63 amino acids (corresponding to amino acids 597 to 659 in the hypothetical yeast protein). On the other side of the insertion there is a stretch of 15 consecutive amino acids that are identical between the yeast protein and the *M. grisea* gene (corresponding to amino acids 746 to 759 in the hypothetical yeast protein). A truncated cDNA homologous to a comple-

Table 4. Cloned genes^a

PTH	Plasmid rescue	Initial	Smallest	cDNA	Gene disruption	Accession no.
1	pCB821	A8A2	pCB889	pCB1294	pCB951	
2-1	pCB880	A1F3	pCB914	pCB1210	pCB965	AF027979
2-2	pCB1466		pCB914			
3	pCB890	A26C8	pCB903	pCB1110	pCB954	AF027980
4	pCB902	A42F1	pCB985		pCB1030	
8	pCB1463	pCB1199	pCB1199	pCB1219		AF027983
9	pCB1468	pCB1503	pCB1254	pCB1362		AF027981
10	pCB1469	pCB1223	pCB1273	pCB1238		AF027982

^a PTH = name of pathogenicity gene; plasmid rescue = plasmid recovered from genomic DNA of the mutant; Initial = initial complementing vector; Smallest = smallest complementing vector; cDNA = cDNA clone; gene disruption = gene disruption vector for the corresponding PTH gene.

menting plasmid also shows extensive homology to the yeast hypothetical protein. Although we have not sequenced the entire *M. grisea* gene, we conclude that *PTH8* is a homolog of the hypothetical yeast gene predicted in GenBank accession no. S51434.

pth9. Southern hybridization analysis of the *pth9* mutant suggested that it contained two independent integration events (data not shown). Segregation analysis suggested that only one of these events conferred hygromycin resistance (Table 3). CP4111, a progeny of a cross between 4136-4-3 and the original mutant, was selected for further analysis since it contained only one insertion, was hygromycin resistant, and showed a disease phenotype similar that of to the original mutant. Sequence from the complementing plasmid, the insertion site, and a cDNA shows that *PTH9* encodes a neutral trehalase homolog. The cDNA for *pth9* has an ORF encoding a protein of 736 amino acids with 54% identity to yeast neutral trehalase (GenBank accession no. X65925).

Fig. 2. Maps of the genomic region of *PTH1*, *PTH2*, *PTH3*, and *PTH4*. Horizontal arrow indicates position of gene. Open bar indicates smallest complementing genomic fragment. Restriction enzymes at edges of this open bar indicate fragment subcloned in the smallest complementing plasmid. Entire bar, including open and solid portions, indicates fragment used to construct gene disruption vector; restriction enzymes at edges of this bar indicate enzymes used to linearize vector for gene disruption transformation. Underlined restriction enzyme sites indicate restriction sites used to insert hygromycin resistance gene in constructing gene disruption vector. Large downward-pointing arrow shows position of insertion of the transforming DNA. For *PTH2* there were two independent insertions in this gene corresponding to *pth2-1* (left) and *pth2-2*. Enzymes marked with an asterisk were used in restriction enzyme-mediated transformation. For all four integration events depicted here by downward arrows, a restriction site for the enzyme used in the transformation is found at the point of integration. The *pth1* mutant was produced without restriction enzyme-mediated transformation. Enzyme abbreviations: B = *Bam*HI; Bc = *Bcl*I; E = *Eco*RI; Ea = *Eag*I; H = *Hind*III; O = *Xho*I; Ml = *Mlu*I; N = *Nor*I; Nc = *Nco*I; S = *Sal*I; T2 = *Sst*II; V = *Eco*RV; Xb = *Xba*I.

pth10. The *pth10* mutant was found in the initial rapid infection assay as a strain that produced very few lesions, but eventually was shown to cause near-wild-type levels of disease in whole plant assays. This mutant produced very few conidia when grown on oatmeal agar in culture, which probably explains why it produced few lesions in the rapid infection assay where conidia were not counted. For example, counts of conidia harvested semi-quantitatively for infection assays showed that cultures of wild-type 4091-5-8 or the *pth10* mutant complemented with pCB1273 (Table 4) generally yielded conidia at 5×10^6 per ml while the *pth10* mutant only yielded 1×10^5 per ml. The conidiation defect was seen on oatmeal plates as a flat-growing culture (data not shown) and this phenotype was used to score progeny and follow complementation. The conidia from *pth10*, how-

```

1 .....MASGSKRSSSS 11
1 MRICHSRTLSNLKDLPTISRRAMHSAIVNSTQKAQFPVETNNGEHYWAE 50
12 LPAGYVEDKS...KGPMRLRF...QESLRLPVPTLEETAARYLRTLKPLLS 56
   | : : | : : | : : | : : | : : | : : | : : | : : | : : | : : |
51 KPNKFYQNKRPNFQG...ITFAKQQLPSLPVPELSTLDKYLQTIKPFPCN 98
   | : : | : : | : : | : : | : : | : : | : : | : : | : : | : : |
57 KEELANSTKAV...QEFVKPGGVGAKLQEKLIARREDPKHKNNWYEWNEAA 105
   | : : | : : | : : | : : | : : | : : | : : | : : | : : | : : |
99 DVETFERQQLCKDFSE...HMGPIQLQRLKEYAND.K.RNWMKAFWDEQS 144
   | : : | : : | : : | : : | : : | : : | : : | : : | : : | : : |
106 YLAYRDPVVPVVSFYFYSHRD...DRRRRDPKRRRAITTAALFEFKMV 150
   | : : | : : | : : | : : | : : | : : | : : | : : | : : | : : |
145 YLQNDPIVPVVSFYFYSHMLPNHLSKIDNDPLIKATAISTVVKFIEAI 194
   | : : | : : | : : | : : | : : | : : | : : | : : | : : | : : |
151 DSGTLEPEYMKKLPICMDSYKWMFNASRVAAPADYP...VKFEAAQHKY 197
   | : : | : : | : : | : : | : : | : : | : : | : : | : : | : : |
195 KDESPLVEIIGKMPFCMNSFLMFTSRLPGKPEDNQDTNIFYSVYENNF 244
   | : : | : : | : : | : : | : : | : : | : : | : : | : : | : : |
198 IVAIRNDRFYKI...EHEVGGRQLNTSELAQFRVYELAGQEAKEP...A 243
   | : : | : : | : : | : : | : : | : : | : : | : : | : : | : : |
245 VTIAYGKFKYKLMTHDGNHKLSENEI...WRQLYSVVFQGSQSDPKLGG 291
   | : : | : : | : : | : : | : : | : : | : : | : : | : : | : : |
244 VGALTSENDRDVTDARALLSAGPANKAALETIEASSFVCLD.DAAPVT 292
   | : : | : : | : : | : : | : : | : : | : : | : : | : : | : : |
292 IGSLSLPRDQWREVMELMK.DPISQDSLETIHKSSFMLCLDLQSPVT 340
   | : : | : : | : : | : : | : : | : : | : : | : : | : : | : : |
293 LEERAHAYWHGDGQNRWYDKPLQFIVNDNGTSGFMGEHSMMDGTPHRLN 342
   | : : | : : | : : | : : | : : | : : | : : | : : | : : | : : |
341 LEEKSRNCWHGDGINRFYDKSLQLVTGNGSSGFLAEHSMKMDGTPPLFLN 390
   | : : | : : | : : | : : | : : | : : | : : | : : | : : | : : |
343 DYVNDIAVNNKLDSD...PSVRSGLPDPSPVRFTVNEELQSEIDR 385
   | : : | : : | : : | : : | : : | : : | : : | : : | : : | : : |
391 NYVQCQL...NKLDVDDFMRKVITPSSSTVAM.KPMELPFIITPKIKHAIES 437
   | : : | : : | : : | : : | : : | : : | : : | : : | : : | : : |
386 AKRDFDAVIGQHELAVQSYQGYGKGLIKKFRCPDAYVQMVQLAYHKMY 435
   | : : | : : | : : | : : | : : | : : | : : | : : | : : | : : |
438 AQLQFKETIGEHLRVVWHYNYGKTFIKRHGMSPDATIQVQLAVFK.Y 486
   | : : | : : | : : | : : | : : | : : | : : | : : | : : | : : |
436 GKNR.PTYESAATRFQQGRTECSRVSSESVAVWCNAMADASAQDADKVK 484
   | : : | : : | : : | : : | : : | : : | : : | : : | : : | : : |
487 LKRQLPTYEAASTRKYFKGRTETGRSVSTASLEFVSKWQNGDVPFAEKIQ 536
   | : : | : : | : : | : : | : : | : : | : : | : : | : : | : : |
485 LFRAAIDSHVEYITAASDGKGVDRHLFGLKRLLEPG.QAVFEIYQDPAYS 533
   | : : | : : | : : | : : | : : | : : | : : | : : | : : | : : |
537 ALKHSAKEHSTYLKNAANGNGVDRHFFGLKNMLKSNDDQIPPLFKDPLFN 586
   | : : | : : | : : | : : | : : | : : | : : | : : | : : | : : |
534 YSSSWYLSLSQLSSEYFNGYGSQVIDAGFGIAYMINENRGLSQLHPSN 583
   | : : | : : | : : | : : | : : | : : | : : | : : | : : | : : |
587 YSSTWLISLSQLSSEYFDGYGSQVNDNGFGLAYMLNNEWL...HI..N 630
   | : : | : : | : : | : : | : : | : : | : : | : : | : : | : : |
584 VIEFATLLTSLLSQHLQHLR.LQGPR..... 608
   | : : | : : | : : | : : | : : | : : | : : | : : | : : | : : |
631 IVNKPA...KSGASVNRHLHYLSQAADFEIDALENENKRKAKL 670

```

Fig. 3. Comparison of the predicted amino acid sequence of Pth2p and the carnitine acetyltransferase gene of *Saccharomyces cerevisiae*. The sequences (upper, *Magnaporthe grisea*) were aligned with the GCG program Bestfit with a gap weight of 2 (Devereaux et al. 1984).

ever, were fully pathogenic in secondary assays in which inoculum levels were standardized. Microscopic examination of lesions produced by the *pth10* mutant showed levels of conidiation indistinguishable from those of wild type (data not shown). Thus, the *pth10* mutation apparently only affects conidiation in culture and not in planta. A cDNA for *PTH10* encodes a 193-amino-acid polypeptide with no significant homology to any known protein.

Disruption of *PTH1*, *PTH2*, and *PTH3* in a rice pathogen.

Strain 4091-5-8, a laboratory strain that is pathogenic on weeping lovegrass and barley, became the strain of choice for our experiments due to its cultural and crossing characteristics and its utility in the rapid barley assay. However, this strain causes no symptoms on rice. We hypothesized that almost all genes required by 4091-5-8 for pathogenicity would be required by all strains of the fungus regardless of host plant species. This hypothesis was tested by performing gene disruptions of *PTH1*, *PTH2*, and *PTH3* in a strain pathogenic on rice. Gene disruption vectors were constructed as detailed in Materials and Methods and as illustrated in Figure 2. These vectors were transformed into protoplasts of CP987, a rice pathogen, and transformants were screened for the mutant phenotype either in culture (*PTH3* for a requirement for histidine) or in infection assays (*PTH1* and *PTH2*). Gene disruption insertion mutants of *PTH1* and *PTH2* in CP987 gave the same pathogenicity defect on rice cv. Yashiro-mochi as that observed with the original mutant on barley (data not shown). The *pth3::Hyg* insertion mutants of CP987 (and of 4091-5-8) showed no growth on minimal medium (Fig. 4) and produced only type 1 flecks in infection assays (data not shown). This more pronounced phenotype on minimal medium and in infection assays is consistent with a complete knockout of the gene as opposed to the promoter insertion in the original mutant.

Fig. 4. Growth of *pth3* mutants and wild type on minimal medium and minimal medium supplemented with histidine. Top row: minimal medium plus 100 mg of histidine per l. Bottom row: minimal medium. Left: 4091-5-8 (wild type). Center: original *pth3* mutant (plasmid insertion in promoter). Right: *pth3::hyg* gene disruption strain (*pth3* null mutant).

DISCUSSION

Molecular genetic investigation of the mechanisms of fungal pathogenesis of plants began in earnest with the development of methods for gene disruption. Production of a null mutation by gene disruption is now the standard for rigorous evaluation of the role of a gene in pathogenesis (Yoder and Turgeon 1997; Oliver and Osbourn 1995; Schafer 1994). For *M. grisea*, gene disruption has been used to evaluate genes involved in signal transduction pathways (Xu et al. 1996, 1997; Mitchell and Dean 1995; Liu and Dean 1997), genes potentially involved in cuticle or cell wall degradation (Sweigard et al. 1992b; Wu et al. 1997), and a gene with high levels of expression during infection (Talbot et al. 1993). In this directed, "reverse genetic" approach mutations are made based on a plausible rationale for the importance of the gene in pathogenicity.

We too have sought genes involved in pathogenicity by performing a more classical, open-ended genetic approach, a mutant hunt. Our method of mutagenesis took advantage of recent developments in fungal transformation to use insertion of transforming DNA as the mutagen. A screen of 5,538 hygromycin-resistant transformants of *M. grisea* has produced 27 mutants, from which eight genes have been cloned. These mutants were termed "*pth*" for pathogenicity. We hoped that this genetic approach ("black box" approach, Oliver and Osbourn 1995) that defines a pathogenic mutant in the broadest possible terms would produce mutations ranging from defects in basic metabolism to defects in genes that are only expressed in a very limited phase of the disease process ("pathogenicity determinants," Oliver and Osbourn 1995; or "pathogenicity genes," Schafer 1994). *PTH3* is an example of a gene involved in basic metabolism, in this case a gene for histidine biosynthesis. Interestingly, the *pth3* mutant shows that even a partial requirement for histidine has a major effect on the ability of *M. grisea* to cause disease. Thus, one of the benefits of a mutant hunt is the identification of potential targets for crop protection chemicals; if the fungus requires the gene to be a robust pathogen then inhibition of the corresponding protein product should control disease. At the other end of the spectrum, we hoped the mutant hunt would identify genes that could be considered "pathogenicity genes" in a stricter sense of the term. *PTH4* is an example of this type of gene. Previously identified as *CpkA* (Mitchell and Dean 1995), this catalytic subunit of a cAMP-dependent protein kinase has been shown to be required for proper appressorium function (Xu et al. 1997) and thus appears to be required for pathogenicity-specific signaling. Based on sequence analysis, all of the other cloned genes that are required for full pathogenicity have homologs in other organisms and/or have a suggested role in metabolism. Thus, they could not strictly be considered pathogenicity determinants.

Though this mutational approach should yield many interesting genes, it also has limitations. All the mutants must be viable in culture and must be able to conidiate, since transformant purification and the infection assay require conidia. Conidiation mutants of *M. grisea* have been obtained by other means (Shi and Leung 1994, 1995). Also, though we attempted to sample all hygromycin-resistant transformants, this process was no doubt biased against viable but slow-growing mutants. Finally, our primary infection assay cannot detect

small differences in disease levels so that we have selected for mutations having a relatively large effect.

In addition to these limitations of insertional mutagenesis we have discovered several cautions. Many transformants with unstable disease phenotypes were found. Transformants of this type were about equal to the number of stable transformants that were further analyzed. They were eliminated from consideration during transformant processing, which included isolation of single conidia and repeated infection assays. Also, one third of the mutants were not tagged by hygromycin resistance. Segregation data did not indicate the presence of additional hygromycin resistance genes. Southern analysis suggests that the trivial explanation, an additional insertion that does not confer hygromycin resistance, is not valid (J. A. Sweigard and A. M. Carroll, *unpublished data*). For REMI transformation, restriction enzyme cutting of the chromosome and imperfect repair provide a means of mutation. For non-REMI transformants, the explanation may be due to the fact that competent cells, by nature of their competency for transformation, may have multiple chromosome breaks that lead to untagged mutations. These mutations may still prove valuable. Those with a symptomless phenotype or a tight, small lesion phenotype could be cloned by complementation by screening of pooled transformants. Those that produce low numbers of normal lesions would require screening of individual transformants if complementation was attempted. If REMI transformation produces deletions, then these deleted sequences could be cloned by subtraction hybridization (Kang et al. 1994). Even though these untagged mutations could potentially be cloned, the ability to verify tagging by segregation of analysis is very valuable in avoiding pursuit of untagged mutations.

Another caution related to insertional mutagenesis concerns deletions or DNA rearrangements that occur during integration. We chose to initially complement the mutations with cosmids rather than plasmids since we were concerned that large deletions of genomic DNA might occur at the integration site; the larger insert size in cosmids would more easily span these deletions to allow complementation. Among the nine insertion events analyzed so far, none underwent a large deletion and all these insertion mutants were easily complemented with plasmids. Recently, however, we have found large deletions (>10 kb) in *pth11-1* and *pth12* (J. A. Sweigard and A. M. Carroll, *unpublished data*). Large deletions were also seen at the *Tox1* locus (Olen Yoder, *personal communication*).

A final caution concerns the choice of REMI transformation for part of the mutant hunt. Though REMI can increase transformation efficiency (Lu et al. 1994; Redman and Rodriguez 1994; Shi et al. 1995) we chose REMI, not to increase transformation efficiency, but to potentially randomize integration sites. The reasoning behind this choice was straightforward: if transformation is limited by the occurrence of chromosomal breaks that provide sites for integration then these breaks might occur nonrandomly at certain hot spots in the genome, while REMI transformation might provide additional, quasi-random breaks that would be more conducive to a tagging approach. Nonrandom integration has been reported in *Aspergillus nidulans* (Diallinas and Scazzocchio 1989) for normal transformation. Our data suggest that restriction enzyme-catalyzed integration is also not random. Among eight genes studied in detail so far, two insertions occurred in the same

gene (*pth2-1* and *pth2-2*). Since it would take 10^5 transformants to saturate the genome with insertions (Kang and Metzberg 1993), multiple hits in the same gene are unlikely with only 5,538 mutants screened. These and other data (Sweigard et al. 1998), including another example of multiple insertions in the same gene, suggest that REMI-based insertions are not random in *M. grisea*.

This mutant hunt has demonstrated the benefits of REMI mutagenesis in *M. grisea* for rapid cloning of genes, compared with chemical mutagenesis followed by map-based cloning or mutant complementation. The major drawback of the technique in *M. grisea* is the apparent nonrandomness of integration sites. For wide-ranging mutant hunts like the one we have performed the benefits of REMI probably outweigh this disadvantage. For mutant hunts aimed at a small number of genes, insertional mutagenesis is probably not the method of choice.

MATERIALS AND METHODS

Fungal strains and media.

The fungal strains used in this study were 4091-5-8 (*MAT1-2*; Valent et al. 1986), 4136-4-3 (*MAT1-1*; Valent and Chumley 1987), 6043 (Leung et al. 1988), and CP987 (*MAT1-1*; Sweigard et al. 1995). Sexual crosses and sporulating cultures were produced on oatmeal agar medium (Crawford et al. 1986).

Fungal transformation.

Transformation and co-transformation were performed as described previously (Sweigard et al. 1995) except that the polyethylene glycol solution was 40% PEG 8000, 20% sucrose, 50 mM KCl, 50 mM NaCl, 10 mM MgCl₂, 50 mM Tris-HCl, pH 8.0. Restriction enzymes (10 to 50 units) were added to some transformations after incubation of protoplasts with DNA and immediately prior to addition of the polyethylene glycol solution. In cases where restriction digests were used as a source of transforming DNA, no attempt was made to destroy the restriction enzyme activity. These reactions were typically 30 μ l with 5 μ g of DNA and 30 units of enzyme.

Transformants generated for the mutant hunt were processed as follows. Transformants were transferred to oatmeal agar with 100 μ g of hygromycin per ml and allowed to conidiate overnight. Conidia were then spread on 2YEG media (2 g of yeast extract per l; 10 g of glucose per l) and allowed to germinate overnight. A single spore from each transformant was then picked to 24-well trays (Falcon 3047) containing oatmeal agar with 100 μ g of hygromycin per ml. These trays were held in the light to allow sporulation and then used as inoculum for the "rapid" infection assay.

Bacterial strains, cosmids, lambda vectors, cDNA libraries, and plasmids.

Plasmids pCB725, pCB819, and pCB1004 (Table 1) were used to generate hygromycin-resistant transformants. The 4392-1-6 cosmid library has been described (Sweigard et al. 1995). Lambda Barfix is a lambda replacement vector that allows cre-lox excision of the genomic DNA insert in a plasmid with the bialaphos resistance gene. It was constructed by ligating *NorI*-digested pBARGEM7-2 (Pall and Brunelli 1993)

into *NotI*-digested Lambda Fix II. Lambda Fix II was purchased from Stratagene (La Jolla, CA). The lambda Barfix library was constructed with 4091-5-8 genomic DNA, following the manufacturer's instructions for Lambda Fix II library construction (Stratagene). Plasmids were excised from phage in CB1180, a λ KC (Elledge et al. 1991) lysogen of DH5 α . A cDNA library was constructed from mRNA prepared from strain 6043 in lambda ZIPLOX according to the manufacturer's instructions (Bethesda Research Labs, Gaithersburg, MD).

Gene replacement vectors were constructed as follows. These constructions are illustrated in Figure 2. *PTH1*: The *SalI* and *BamHI* sites of pBluescript KSII⁺ were eliminated by restriction digestion with these enzymes, followed by Klenow fill-in and religation to produce pCB943. The 6-kb *XhoI* fragment of pCB876 was ligated into pCB943 to produce pCB947. The 1.4-kb *SalI/BamHI* fragment of pCSN43, containing the hygromycin resistance gene, was ligated into the *SalI/BamHI* sites of pCB947 to produce pCB951. *PTH2*: Plasmid pCB946 was digested with *EagI* and *NcoI* and the *NotI/NcoI* (partial) of pCB952 containing the hygromycin resistance gene was ligated into these sites. *PTH3*: Plasmid pCB948 was digested with *EagI*, treated with Klenow, and ligated to the 1.4-kb *SalI/BamHI* fragment of pCSN43 (Klenow-treated) to produce pCB954. *PTH4*: Plasmid pCB945 was digested with *EagI* and *EcoRV* and ligated to the *XbaI* (Klenow-treated)/*NotI* fragment of pCB952 to produce pCB1030. This gene disruption vector was used by Xu et al. (1997).

Genomic DNA surrounding the insertion site of transforming DNA was recovered by "plasmid rescue" as follows. Genomic DNA (1 to 3 μ g) was digested with a restriction enzyme that did not cut in the transforming vector or only cut the vector once in a region not required for replication in *Escherichia coli*. The digested DNA was ethanol precipitated and ligated in a 100- μ l ligation reaction. Four microliters of this ligation mix was transformed into *E. coli* strain DH5 α (Hanahan 1983) or DH10B (Bethesda Research Labs) by chemical transformation or electroporation.

Infection assays.

The "standard" infection assay was performed as described previously (Valent et al. 1991) by spraying conidia on weeping lovegrass (*Eragrostis curvula*) (2×10^6 conidia total), barley (*Hordeum vulgare*) cv. Bonanza (1×10^6 conidia total), and rice (*Oryza sativa*) cv. Yashiro-mochi (5×10^5 conidia total). Lesions in these assays were scored according to the rating system described by Valent et al. (1991). Most transformants were first analyzed in a primary "rapid" assay on barley ("cotton swab assay"). For this screen a cotton swab was wetted with a 0.25% gelatin (Type B, Sigma, St. Louis, MO) solution and rubbed on a sporulating culture to collect conidia. The swab was then rubbed over 2 to 3 leaves of 7-day-old barley. The plants were examined 5 days after inoculation and those that showed reduced symptoms were tested in a standard infection assay with conidia sprayed at controlled inoculum concentrations.

Recombinant DNA techniques.

Restriction enzymes (Bethesda Research Labs) and T4 DNA ligase (New England Biolabs, Boston, MA) were used

according to the manufacturers' instruction. Isolation of fungal genomic DNA and bacterial plasmids, oligolabeling of probes, gel electrophoresis, gel blotting, and blot hybridizations were performed as described previously (Sweigard et al. 1992a).

ACKNOWLEDGMENTS

We thank Phil Smith and Kathy Kline-Smith for providing plants, and Amity Woolard and Kathy Kline-Smith for assistance in picking transformants.

LITERATURE CITED

- Bailey, R. B., and Woodward, A. 1984. Isolation and characterization of a pleiotropic glucose repression resistant mutant of *Saccharomyces cerevisiae*. *Mol. Gen. Genet.* 193:507-512.
- Bölker, M., Böhnert, H. U., Braun, K. H., Görl, J., and Kahmann, R. 1995. Tagging pathogenicity genes in *Ustilago maydis* by restriction enzyme-mediated integration (REMI). *Mol. Gen. Genet.* 248:547-552.
- 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.
- Devereux, J., Haeblerli, P., and Smithies, O. 1984. A comprehensive set of sequence analysis programs for the VAX. *Nucleic Acids Res.* 16:387-395.
- Diallinas, G., and Scazzocchio, C. 1989. A gene coding for the uric acid-xanthine permease of *Aspergillus nidulans*: Inactivational cloning, characterization, and sequence of a *cis*-acting mutation. *Genetics* 122:341-350.
- Elledge, S. J., Mulligan, J. T., Ramer, S. W., Spottswood, M., and Davis, R. W. 1991. λ YES: A multifunctional cDNA expression vector for the isolation of genes by complementation of yeast and *Escherichia coli* mutants. *Proc. Natl. Acad. Sci. USA* 88:1731-1735.
- Flick, J. S., and Johnston, M. 1991. *GRR1* of *Saccharomyces cerevisiae* is required for glucose repression and encodes a protein with leucine-rich repeats. *Mol. Cell. Biol.* 11:5101-5112.
- Hanahan, D. 1983. Studies on transformation of *Escherichia coli* with plasmids. *J. Mol. Biol.* 166:557-580.
- Howard, R. J., and Valent, B. 1996. Breaking and entering: Host penetration by the fungal rice blast pathogen *Magnaporthe grisea*. *Annu. Rev. Microbiol.* 50:491-512.
- Kang, S., Chumley, F. G., and Valent, B. 1994. Isolation of the mating-type genes of the phytopathogenic fungus *Magnaporthe grisea* using genomic subtraction. *Genetics* 138:289-296.
- Kang, S., and Metzenberg, R. L. 1993. Insertional mutagenesis in *Neurospora crassa*: cloning and molecular analysis of the *preg+* gene controlling the activity of the transcriptional activator *NUC-1*. *Genetics* 133:193-202.
- 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.
- 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.
- Liu, S., and Dean, R. A. 1997. G protein α subunit genes control growth, development, and pathogenicity of *Magnaporthe grisea*. *Mol. Plant-Microbe Interact.* 10:1075-1086.
- Lu, S., Lyngholm, L., Yang, G., Bronson, C., Yoder, O. C., and Turgeon, B. G. 1994. Tagged mutations at the *Tox1* locus of *Cochliobolus heterostrophus* by restriction enzyme-mediated integration. *Proc. Natl. Acad. Sci. USA* 91:12649-12653.
- Mitchell, T. K., and Dean, R. A. 1995. The cAMP-dependent protein kinase catalytic subunit is required for appressorium formation and pathogenesis by the rice blast pathogen *Magnaporthe grisea*. *Plant Cell* 7:1869-1878.
- Oliver, R., and Osbourn, A. 1995. Molecular dissection of fungal phytopathogenicity. *Microbiology* 141:1-9.
- Pall, M. L., and Brunelli, J. P. 1993. A series of six compact fungal transformation vectors containing polylinkers with multiple unique restriction sites. *Fungal Genet. Newsl.* 40:59-62.

- Redman, R. S., and Rodriguez, R. J. 1994. Factors affecting the efficient transformation of *Colletotrichum* species. *Exp. Mycol.* 18:230-246.
- Schafer, W. 1994. Molecular mechanisms of fungal pathogenicity to plants. *Annu. Rev. Phytopathol.* 32:461-477.
- 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.
- Shi, Z., Christian, D., and Leung, H. 1995. Enhanced transformation in *Magnaporthe grisea* by restriction enzyme mediated integration of plasmid DNA. *Phytopathology* 85:329-333.
- Shi, Z., and Leung, H. 1994. Genetic analysis and rapid mapping of a sporulation mutation in *Magnaporthe grisea*. *Mol. Plant-Microbe Interact.* 7:113-120.
- Shi, Z., and Leung, H. 1995. Genetic analysis of sporulation in *Magnaporthe grisea* by chemical and insertional mutagenesis. *Mol. Plant-Microbe Interact.* 8:949-959.
- Sweigard, J. 1996. A REMI primer for filamentous fungi. *IS-MPMI-Rep. Spring* 1996, pp. 3-5.
- Sweigard, J. A., Carroll, A. M., Kang, S., Farrall, L., Chumley, F. G., and Valent, B. 1995. Identification, cloning, and characterization of *PWL2*, a gene for host species specificity in the rice blast fungus. *Plant Cell* 7:1221-1233.
- Sweigard, J. A., Carroll, A. M., and Valent, B. 1998. Restriction enzyme-mediated integration in the rice blast fungus. *Long Ashton Int. Symp., 15th. IACR-Long Ashton Res. Sta., Bristol, England.*
- Sweigard, J. A., Chumley, F. G., and Valent, B. 1992a. Cloning and analysis of *CUTI*, a cutinase gene from *Magnaporthe grisea*. *Mol. Gen. Genet.* 232:174-182.
- Sweigard, J. A., Chumley, F. G., and Valent, B. 1992b. Disruption of a *Magnaporthe grisea* cutinase gene. *Mol. Gen. Genet.* 232:183-190.
- Talbot, N. J., Ebbole, D. J., and Hamer, J. E. 1993. Identification and characterization of MPG1, a gene involved in pathogenicity from the rice blast fungus *Magnaporthe grisea*. *Plant Cell* 5:1575-1590.
- Tilburn, J., Roussel, F., and Scazzocchio, C. 1990. Insertional inactivation and cloning of the *wA* gene of *Aspergillus nidulans*. *Genetics* 126:81-90.
- Valent, B., and Chumley, F. G. 1987. Genetic analysis of host species specificity in *Magnaporthe grisea*. Pages 83-93 in: *Molecular Strategies for Crop Protection*. Alan R. Liss, New York.
- Valent, B., and Chumley, F. G. 1991. Molecular genetic analysis of the rice blast fungus, *Magnaporthe grisea*. *Annu. Rev. Phytopathol.* 29:443-467.
- Valent, B., Crawford, M. S., Weaver, C. G., and Chumley, F. G. 1986. Genetic studies of fertility and pathogenicity in *Magnaporthe grisea* (*Pyricularia oryzae*). *Iowa State J. Res.* 60:569-594.
- Valent, B., Farrall, L., and Chumley, F. G. 1991. *Magnaporthe grisea* genes for pathogenicity and virulence identified through a series of backcrosses. *Genetics* 127:87-101.
- Wu, S.-C., Ham, K.-S., Darvill, A. G., and Albersheim, P. 1997. Deletion of two *endo*-B-1,4-xylanase genes reveals additional isozymes secreted by the rice blast fungus. *Mol. Plant-Microbe Interact.* 10:700-708.
- Xu, J.-R., and Hamer, J. E. 1996. MAP kinase and cAMP signaling regulate infection structure formation and pathogenic growth in the rice blast fungus *Magnaporthe grisea*. *Genes Dev.* 10:2696-2706.
- Xu, J.-R., Urban, M., Sweigard, J. A., and Hamer, J. E. 1997. The *CPKA* gene of *Magnaporthe grisea* is essential for appressorial penetration. *Mol. Plant-Microbe Interact.* 10:187-194.
- Yoder, O. C., and Turgeon, B. G. 1997. Molecular-genetic evaluation of fungal molecules for roles in pathogenesis to plants. *Genetics* 75:425-440.
- Yoder, O. C., Valent, B., and Chumley, F. 1986. Genetic nomenclature and practice for plant pathogenic fungi. *Phytopathology* 76:383-385.