Characterization of Mutations in the Beta-Tubulin Gene of Benomyl-Resistant Field Strains of *Venturia inaequalis* and Other Plant Pathogenic Fungi

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ABSTRACT

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All benomyl-resistant field strains of *Venturia inaequalis, V. pirina, Monilinia fructicola, Sclerotinia homoeocarpa*, and six species of *Penicillium*, except those with low resistance to benomyl, were found to contain a single base pair mutation in their beta-tubulin gene that resulted in an amino acid substitution in beta-tubulin. In *V. inaequalis*, codon 198, which encodes glutamic acid in a sensitive strain, was converted to a codon for alanine in a strain with very high resistance, to a codon for lysine in a strain with high resistance, or to a codon for glycine in a strain with medium resistance to benomyl. Codon 200 for phenylalanine was converted to a codon for tyrosine in a second strain of *V. inaequalis* with medium resistance to benomyl. Among field strains of other fungi, 14 had a glutamic acid to lysine, alanine, or valine substitution at position 198, and three had a phenylalanine to tyrosine substitution at position 200. Among seven benomyl-resistant strains with sensitivity to the *N*-phenylcarbamate fungicide diethofencarb, all had a glutamic acid to alanine or glycine substitution at position 198. A comparison of the codon changes in the beta-tubulin gene of field strains with laboratory-induced benomyl-resistant mutants of model fungi showed that mutations conferring field resistance represent a small subset of the mutations recovered in laboratory experiments.

Benomyl-resistant *Venturia inaequalis* (Cooke) G. Wint. isolates were detected shortly after intensive and exclusive use of benomyl in Michigan apple orchards (11) and in most countries where benzimidazole fungicides were used to control apple scab. In *V. inaequalis*, resistance to benomyl was attributed to mutations in a single gene with different alleles conferring widely different levels of resistance (28,33). Sensitivity of *V. inaequalis* to *N*-phenylcarbamates, a group of mitosis-inhibiting fungicides, was found to be negatively correlated with very high resistance (VHR) to benomyl. Sensitivity to the *N*-phenylcarbamate fungicides diethofencarb and methyl-*N*-(3,5-dichlorophenyl) carbamate (MDPC) was also attributed to a single gene, and it was postulated that the VHR allele for resistance to benomyl was identical with the allele for sensitivity to diethofencarb (10,29).

The characterization of laboratory-induced resistant mutants in model organisms like *Drosophila, Aspergillus*, and *Arabidopsis* has become a common approach for studying resistance mechanisms to pesticides (5,7,12–14). In binding studies (3), beta-tubulin from a benomyl-resistant strain of *Aspergillus nidulans* exhibited reduced affinity for carbendazim, the toxic conversion product of benomyl. Later, molecular characterization of the beta-tubulin gene of benomyl-resistant mutants of *A. nidulans* (12,13), *Neurospora crassa* (6,25), and *Saccharomyces cerevisiae* (34) provided evidence that point mutations in this gene were responsible for resistance to benomyl. A point mutation in the beta-tubulin gene was also found in a benomyl-resistant strain of *N. crassa* with increased sensitivity to diethofencarb, and it was postulated that diethofencarb could bind to the altered carbendazim-binding site (6). Whether these same mutations were responsible for the development of benomyl-resistant plant pathogenic fungi has not been determined.

This study was conducted to determine the molecular basis for resistance to benomyl in field strains of *V. inaequalis* with widely different levels of resistance to benomyl and sensitivity to *N*-phenylcarbamates. The basis for benomyl resistance in field strains of other plant pathogens was investigated to establish whether mutations associated with resistance in these fungi were identical to the mutations found in *V. inaequalis*. Mutations in the beta-tubulin gene of field strains were compared with mutations reported for laboratory-induced strains of model fungi to determine whether the same mutations were involved in field- and laboratory-induced resistance to benomyl.

MATERIALS AND METHODS

Fungal strains. The single-spore isolates of *V. inaequalis* were from a worldwide collection of field strains previously characterized in studies on the inheritance of resistance to benomyl and *N*-phenylcarbamates (10,33). Isolates of several species of *Penicillium* were provided by D. A. Rosenberger, New York State Agricultural Experiment Station, Cornell University, Geneva; those of *V. pirina* Aderhold, by E. Shabi, Agricultural Research Organization, The Volcani Center, Bet Dagan, Israel; those of *Monilinia fructicola* (G. Wint.) Honey, by A. L. Jones; and those of *Sclerotinia homoeocarpa* F. T. Bennett, by J. M. Vargas, Michigan State University, East Lansing. All of the strains were field isolates. The cultures were maintained on Difco potato-dextrose agar (PDA).

The reaction of the strains to benomyl (Benlate 50WP; E. I. du Pont de Nemours & Co., Wilmington, DE) and to MDPC and diethofencarb (Sumitomo Chemical Company, Ltd., Takatsukasa Takarazuka, Japan) was determined by transferring pieces of mycelium to PDA amended with benomyl at 0, 0.5, 5, and 50 mg/L, MDPC at 2.5 mg/L, or diethofencarb at 0.5 mg/L. The strains were then classified as established in previous studies on benomyl resistance (10,15,28–30) into one of the following phenotypes: sensitive (S), with no growth on 0.5 mg/L benomyl; low resistance (LR), with growth on 0.5 mg/L but not 5 on mg/L; medium resistance (MR), with growth on 5 mg/L but not on 50 mg/L; high resistance (HR), with slow growth on 50 mg/L; and very high resistance (VHR), with rapid growth on 50 mg/L. Strain I-26 of *V. inaequalis*, previously reported as atypical for MR isolates because of its unusual sensitivity to diethofencarb (9,27), was assigned the phenotype MR−.

Bacteria and plasmids. *Escherichia coli* strain LE 392 (23) was used as a host for phage replication. Plasmids pUC19 and pUCBM20 (Boehringer Mannheim, Indianapolis, IN) were used for cloning experiments and were propagated in *E. coli* strains DH5α and HB101 in the presence of ampicillin at 50 mg/L.

Cloning the beta-tubulin gene from *V. inaequalis*. Agar plugs with actively growing mycelium of the benomyl-sensitive strain WC or the VHR strain KV3C were fragmented in sterile distilled water with a tissue grinder (Cat. No. 7727; Corning Glass Works, Corning, NY) and added to 200 ml of Difco potato-dextrose broth (PDB) in 1-L flasks coated with dichlorodimethylsilane. The cultures were incubated on a rotary shaker at 100 rpm for 3 wk at about 20 C. The mycelium was harvested by filtration of medium through Miracloth (Calbiochem Corp., La Jolla, CA). The mycelium (5–10 g wet weight) was rinsed with distilled water, dried between paper towels, and then frozen with liquid nitrogen in a mortar. Frozen mycelium was ground to a powder, suspended by vortexing in 50 ml of lysis buffer (100 mM LiCl, 50 mM Na_2EDTA, 1.0% SDS, 10 mM Tris-HCl, pH 7.4) with 20 μg/ml of proteinase K (Boehringer Mannheim) and then incubated at 50 C for 1 h. The DNA was purified by phenol extraction and ethanol precipitation. The pellet was resuspended in 4 ml of TE buffer (10 mM Tris-HCl, pH 7.4, 1 mM EDTA), and mixed with 4.2 g of CsCl and 0.17 ml of ethidium bromide (10 mg/ml). The solution was centrifuged in a Sorvall TV-865 rotor (Du Pont) for 14 h at 220,000 *g*. The DNA was partially digested with *Sau*3A (Boehringer Mannheim) and size-fractionated on a 10–40% sucrose gradient. DNA fragments, 16–20 kb, were ligated into dephosphorylated, *Bam*HI-digested, EMBL3 arms according to the instructions of the manufacturer (Promega, Madison, WI). The ligation mixtures were packaged in vitro with Packagene lambda DNA packaging system (Promega), and the resulting phages were used to transfect *E. coli* LE 392.

The beta-tubulin gene cloned from *Erysiphe graminis* DC. f. sp. *hordei* Ém. Marchal (31) was used as a probe to screen the libraries. Two internal *Hin*dIII DNA fragments, encoding codons 22–428 of the *E. g. hordei* beta-tubulin gene, were labeled with α-^{32}P-dCTP (Du Pont, Boston, MA) using the random primed labeling method (4). Hybridization was performed in a solution of high ionic strength (6× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 5× Denhardt's reagent, 0.5% SDS, and 100 μg/ml denatured salmon sperm DNA) (20) at 55 C for 12 h. Filters were then washed twice in each of the following solutions: 2× SSC at 20 C for 5 min, 2× SSC and 1% SDS at 55 C for 30 min, and 0.1× SSC at 20 C for 30 min. Recombinant phage DNA that hybridized with the heterologous beta-tubulin probe was digested with the restriction enzymes *Bam*HI, *Bgl*I, *Eco*RI, *Sal*I, *Xba*I, and *Xho*I (Boehringer Mannheim) and subjected to Southern analysis (32).

Sequence analysis of the beta-tubulin gene from *V. inaequalis*. Unidirectional deletions of the beta-tubulin gene of strain WC and strain KV3C were obtained with the Erase-a-Base system (Promega) according to the protocol of the manufacturer. Sequencing of insert DNA was performed by the dideoxy chain termination method (27) with Sequenase (United States Biochemical, Cleveland, OH). Alkaline-denatured double-stranded plasmid DNA was used as template in the sequencing reactions (35). Both strands of the coding region and 300 bp of the upstream and downstream flanking regions of the beta-tubulin genes were sequenced.

Rapid cloning of the beta-tubulin DNA with the polymerase chain reaction. Actively growing mycelium was fragmented with a tissue grinder and added to 50 ml of PDB in siliconized 250-ml flasks. The cultures were shaken at 100 rpm at about 20 C for 1–5 days. DNA samples were prepared as described above, except that the final step, CsCl equilibrium density centrifugation, was omitted.

The polymerase chain reaction (PCR) and then sequence analysis of the cloned DNA fragment were used to rapidly characterize the sequence of beta-tubulin DNA from a variety of strains. Genomic DNA was prepared from each strain of *V. inaequalis* and subjected to PCR, in which a 22-mer oligonucleotide A (5′-CAAACCATCTCTGGCGAACACG) and a 22-mer oligonucleotide B (5′-TGGAGGACATCTTAAGACCACG) were used as primers. Primer A was identical in sequence to codons 22–28, and primer B was complementary in sequence to codons 359–365 of the beta-tubulin gene of *V. inaequalis* (Fig. 1). With these primers a 1,191-bp fragment of the beta-tubulin gene was amplified. A *Bam*HI restriction site, immediately downstream from the annealing site of primer A, and a *Bfr*I restriction site in primer B (Fig. 1), were used to subclone the amplified beta-tubulin DNA.

Genomic DNA prepared from each of the other fungal species was subjected to PCR by using two generic beta-tubulin primers. Constraints on primer design were that the amplified DNA had to contain codons 167 and 241, in which mutations were associated with resistance to benomyl (25,34), and that the primers had to anneal to a conserved region with minimal variation in the sequence. A 24-mer oligonucleotide C (5′-GAGGAATTCC-CAGACCGTATGATG) and a 28-mer oligonucleotide D (5′-GCTGGATCCTATTCTTTGGGTCGAACAT) were chosen as generic beta-tubulin primers. Primer C was nearly identical in sequence to codons 157–164, and primer D was complementary in sequence to codons 293–300 of the beta-tubulin gene (Fig. 1). PCR with these primers was expected to amplify a 436-bp fragment of the beta-tubulin genes. Primers C and D contained an *Eco*RI and *Bam*HI restriction site, respectively, to facilitate subcloning of amplified DNA (Fig. 1). The primers were synthesized in the Macromolecular Structure Facility, Department of Biochemistry, Michigan State University, East Lansing.

Reactions were performed in a thermal cycler (Perkin-Elmer, Norwalk, CT) with the Repliprime DNA amplification system (Du Pont) according to the manufacturer's procedures. Negative controls were run in all the amplification reactions to detect contamination. In reactions involving primers A and B, 35 cycles were performed for each reaction as follows: 94 C, 1 min; 55 C, 1 min; and 72 C, 2 min. In reactions involving primers C and D, the annealing temperature was reduced from 55 to 50 C. Amplification products were analyzed for the expected 1,191-bp or 436-bp fragments by agarose gel electrophoresis in 1× TBE buffer (0.1 M Tris-HCl, 0.1 M boric acid, 0.02 mM EDTA, pH 8.3). Following electrophoresis, the DNA was visualized with ethidium bromide. The amplified sequences were precipitated, digested with the appropriate restriction enzymes, directionally subcloned into pUC19 or pUCBM20, and the resulting plasmids were used to transform *E. coli* strain DH5α or HB101.

A 300-bp DNA fragment was sequenced with custom-designed beta-tubulin–specific sequencing primers to characterize codons 164–264 in the subcloned DNA fragments from LR, MR, and HR strains of *V. inaequalis*. The subcloned, 436-bp DNA fragments from the other fungal species were sequenced entirely.

DNASIS (Hitachi America Ltd., San Bruno, CA) software was used to analyze and compare the sequences. Open reading frames found in the sequences were compared with previously characterized beta-tubulin genes of other fungi to determine whether the sequences were beta-tubulin sequences. Comparisons of the deduced amino acid sequences from sensitive and benomyl-resistant strains were used to determine the amino acids that were associated with resistance to benomyl. The codon number assignments of partially characterized beta-tubulin sequences of fungi other than *V. inaequalis* were based on homology with other beta-tubulin genes (1). The codon substitutions observed in the field strains were compared with changes reported for laboratory-induced mutants with resistance to benomyl (6,12,13,25,34).

RESULTS

Cloning and sequence analysis of the beta-tubulin gene from *V. inaequalis*. The genomic libraries of the S and VHR strains containing 1×10^4 and 2×10^4 clones, respectively, were screened with the radiolabeled beta-tubulin clone from *E. g. hordei*. DNA from two and eight clones of the S and VHR libraries, respectively, hybridized strongly with the heterologous beta-tubulin DNA probe. In Southern analysis (data not shown), single bands of *Bam*HI-, *Bgl*I-, *Xba*I-, and *Xho*I-digested DNA fragments from

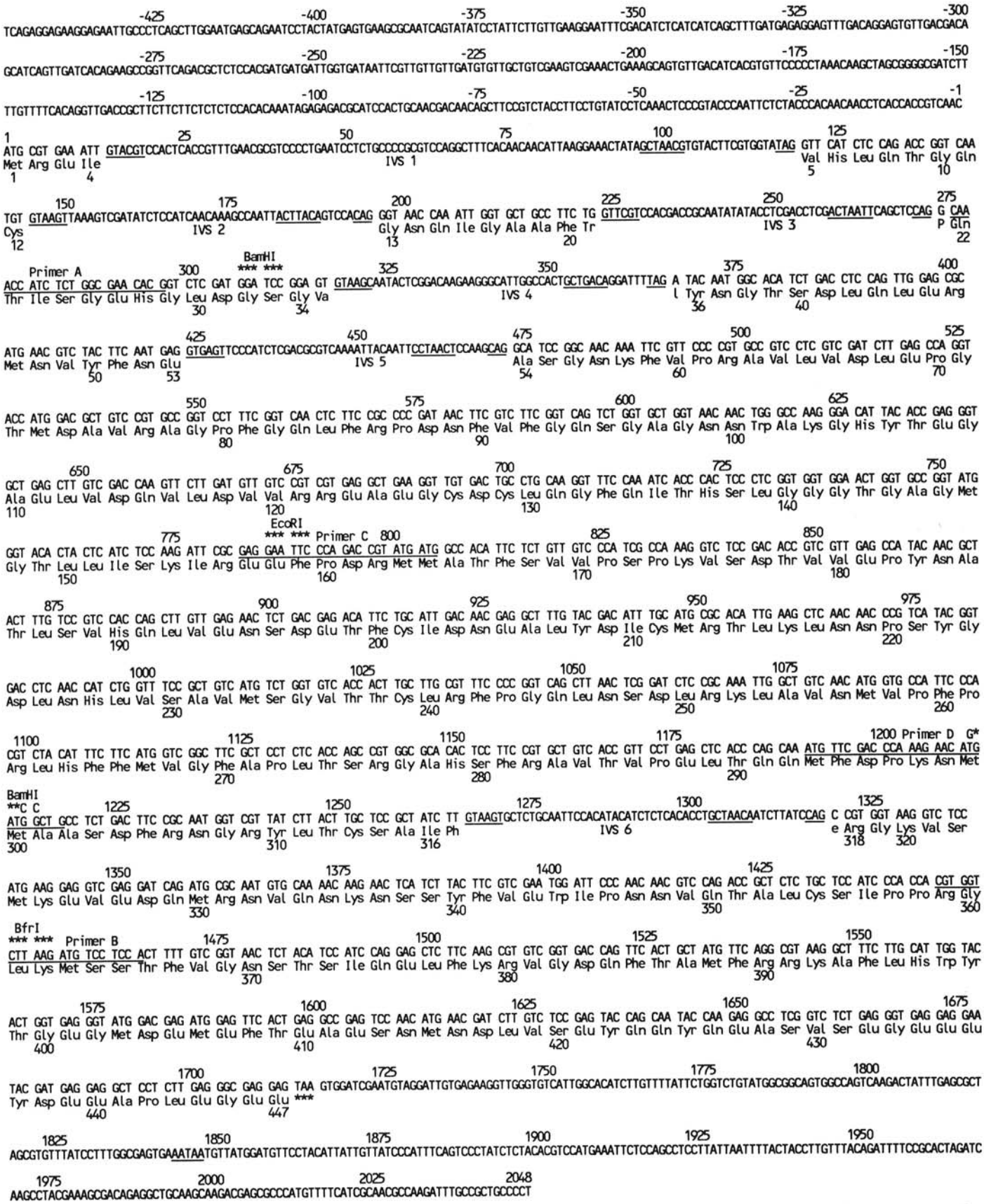

Fig. 1. Nucleotide sequence of the beta-tubulin gene from the benomyl-sensitive strain WC of *Venturia inaequalis*. The nucleotide sequence, starting 428 bp downstream from a *Xho*I site, is numbered from the ATG initiation codon (numbers appear above the nucleotides). The deduced amino acid sequence is indicated by the three-letter amino acid code below the nucleotides (numbers are given below the amino acid sequence). The six intervening sequences are indicated by IVS 1–6. Putative 5′ and 3′ splice junction and lariat formation sequences in IVS 1–6, and a potential polyadenylation sequence in the 3′-flanking region of the gene, are underlined. Annealing sites for primers A–D (see text) are underlined. A *Bam*HI site, directly downstream from primer A, *Bfr*I, *Eco*RI, and *Bam*HI sites in primers B, C, and D, respectively, were used for the directional cloning of amplified DNA and are shown above the nucleotide sequence. Three nonhomologous nucleotides in primer D, as shown in the *Bam*HI sequence, were used to generate a new *Bam*HI site in amplified DNA.

the lambda clones were found to hybridize with the *E. g. hordei* beta-tubulin probe. Additional restriction analysis, in combination with Southern analysis, suggested that a 7.0-kb *Xho*I DNA fragment of the clones contained the entire beta-tubulin, and this DNA fragment was subcloned from one clone of each library into pUC19.

Nucleotide sequence analysis of DNA cloned from the S and VHR strains of *V. inaequalis* revealed the presence of seven exons, encoding a protein of 447 amino acids, and six intervening sequences (IVS 1–6) (Fig. 1). Comparison of the deduced amino acid sequence from the cloned sequence of *V. inaequalis* and the beta-tubulin genes from *E. g. hordei* (31), *N. crassa* (25), and *A. nidulans* (*Ben*A) (21) showed 98, 96, and 96% homology, respectively. In addition, the positions of IVS 1–6 in the cloned sequences of *V. inaequalis* were identical to the positions of IVS 1–6 in the beta-tubulin gene from *E. g. hordei* and *N. crassa* (data not shown). The nucleotide sequence for the beta-tubulin gene of *V. inaequalis* was submitted to the Genbank data base under accession number M97951. In Southern analysis with *Bgl*I-, *Xba*I-, and *Xho*I-digested genomic DNA of strain KV3C, only single bands of DNA with a size of 20, 30, and 7.0 kb respectively, hybridized with the cloned beta-tubulin DNA from *V. inaequalis*.

Only a single codon change was detected when the structural beta-tubulin gene from the VHR strain KV3C was compared with the nucleotide sequence of the beta-tubulin gene cloned from the S strain WC. Codon 198 for glutamic acid (GAG) in the S strain was replaced by a codon for alanine (GCG) in the VHR strain (Table 1).

The 300-bp DNA fragments of PCR amplified DNA from LR, MR, MR−, and HR strains of *V. inaequalis* showed complete or nearly complete sequence identity with the corresponding region in the sequenced beta-tubulin gene from S strain WC. The MR, MR−, and HR strains of *V. inaequalis* exhibited single base pair mutations, but no mutations were observed in the LR strain. The MR strain contained a single base pair mutation converting codon 200 from phenylalanine (TTC) in the benomyl-sensitive strain to a codon for tyrosine (TAC). The MR− and HR strain contained mutations converting codon 198 for glutamic

TABLE 1. Point mutations and deduced amino acid substitutions in the beta-tubulin gene for field strains of several plant pathogenic fungi with resistance to benomyl and sensitivity to methyl-*N*-(3,5-dichlorophenyl)carbamate (MDPC) and diethofencarb (NPC)

Fungal species and strains	Phenotype			Codon substitution	Amino acids in position		
	Benomyl[a]	MDPC[b]	NPC[b]		198	199	200
Venturia inaequalis							
WC	S	IS	IS	None	Glu	Thr	Phe
MINNS 118	LR	IS	IS	None	Glu	Thr	Phe
I-26	MR−	S	S	GAG to GGG	Gly	Thr	Phe
MAINE 8	MR	IS	IS	TTC to TAC	Glu	Thr	Tyr
RH-4	HR	S	IS	GAG to AAG	Lys	Thr	Phe
KV3C	VHR	S	S	GAG to GCG	Ala	Thr	Phe
V. pirina							
IL-7	S	IS	IS	None	Glu	Thr	Phe
IL-8	S	IS	IS	None	Glu	Thr	Phe
IL-2	MR	IS	IS	TTC to TAC	Glu	Thr	Tyr
IL-5	VHR	S	S	GAG to GCG	Ala	Thr	Phe
IL-6	VHR	S	S	GAG to GCG	Ala	Thr	Phe
Monilinia fructicola							
SHANE	S	...[c]	IS	None	Glu	Thr	Phe
CB-2	HR	...	IS	GAA to AAA	Lys	Thr	Phe
Penicillium puberulum							
CO-4	S	IS	IS	None	Glu	Thr	Phe
CO-67	HR	S	IS	GAG to AAG	Lys	Thr	Phe
CO-164	VHR	S	S	GAG to GCG	Ala	Thr	Phe
P. digitatum							
DI-V4	S	IS	IS	None	Glu	Thr	Phe
DI-M18	HR	S	IS	GAG to AAG	Lys	Thr	Phe
DI-M13	HR	S	IS	GAG to AAG	Lys	Thr	Phe
P. expansum							
EX-99	S	S	IS	None	Glu	Thr	Phe
EX-132	LR	IS	IS	None	Glu	Thr	Phe
EX-301	HR	S	IS	GAG to GTG	Val	Thr	Phe
EX-24	VHR	S	S	GAG to GCG	Ala	Thr	Phe
P. italicum							
IT-7E	S	S	IS	None	Glu	Thr	Phe
IT-7G	MR	IS	IS	TTC to TAC	Glu	Thr	Tyr
IT-7M	HR	S	IS	GAG to AAG	Lys	Thr	Phe
P. aurantiogriseum							
SO-198	LR	IS	IS	None	Glu	Thr	Phe
SO-147	MR	IS	IS	TTC to TAC	Glu	Thr	Tyr
SO-126	HR	S	IS	GAG to AAG	Lys	Thr	Phe
SO-298	VHR	S	S	GAG to GCG	Ala	Thr	Phe
P. viridicatum							
VI-227	S	IS	IS	None	Glu	Thr	Phe
VI-77	HR	S	IS	GAG to AAG	Lys	Thr	Phe
Sclerotinia homoeocarpa							
BEN-S	S	...	IS	None	Glu	Thr	Phe
BEN-R	HR	...	IS	GAG to AAG	Lys	Thr	Phe

[a] Benomyl, MDPC, and NPC phenotypes were determined by growing the strains on potato-dextrose agar amended with benomyl at 0, 0.5, 5, and 50 mg/L; MDPC at 2.5 mg/L; and NPC at 0.5 mg/L, respectively. Sensitive (S) = no growth with benomyl at 0.5 mg/L; low resistance (LR) = growth on 0.5 mg/L but not 5 on mg/L; medium resistance (MR) = growth on 5 mg/L but not on 50 mg/L; high resistance (HR) = slow growth on 50 mg/L; very high resistance (VHR) = rapid growth on 50 mg/L.

[b] Insensitive (IS) = rapid growth with MDPC at 2.5 mg/L or with NPC at 0.5 mg/L; sensitive (S) = no growth with MDPC at 2.5 mg/L or NPC at 0.5 mg/L.

[c] Not determined.

acid (GAG) in the S strain to codons for glycine (GGG) and lysine (AAG), respectively (Table 1).

Characterization of beta-tubulin DNA fragments from other fungal species. Although PCR amplification of genomic DNA from other fungal species with the 22-mer primers was possible, directional cloning of the DNA target sequence was inefficient. Restriction site analysis (data not shown) revealed that the *Bam*HI site, situated immediately downstream from primer A in amplified beta-tubulin DNA from strains of *V. inaequalis*, was not conserved in the beta-tubulin DNA of fungi such as *A. nidulans* (21), *E. graminis* (31), and *N. crassa* (25). Directional cloning of PCR products generated with primers C and D was more efficient, since both primers contained restriction sites.

Specific 436-bp DNA fragments were amplified from strains of *V. pirina, M. fructicola*, and *S. homoeocarpa*. All of the cloned DNA fragments showed extensive sequence similarity with the beta-tubulin gene from *V. inaequalis*. A comparison of the deduced amino acid sequence of beta-tubulin of *V. inaequalis* with all other fungal species revealed homologies of higher than 96%. In the strains with resistance to benomyl, single-point mutations at codon 198, converting glutamic acid to lysine or alanine, or at codon 200, converting phenylalanine to tyrosine, were found (Table 1).

Agarose gel electrophoresis showed that a specific, slightly larger DNA fragment was amplified from strains of *Penicillium* species than from strains of the other fungi. Nucleotide sequence analysis of the beta-tubulin DNA fragments cloned from the *Penicillium* strains showed that an additional 51 bp of sequence were present in the PCR-amplified DNA fragments. The extra sequence separated codons 205 and 206 and contained typical fungal intron splice signals (25). The position of this intron was identical to the position of IVS 6 found in the *Ben*A gene of *A. nidulans* (21). Analysis of the DNA from benomyl-resistant and sensitive strains of *Penicillium* showed that point mutations were present in codons 198 or 200 from benomyl-resistant strains as previously found in benomyl-resistant strains of *V. inaequalis*. A new mutation in codon 198 was detected in *P. expansum* strain EX-301 with an HR phenotype. The codon for glutamic acid in the benomyl-sensitive strain was replaced in strain EX-301 with a codon for valine (Table 1).

Field and laboratory-induced mutations in the beta-tubulin gene. A more restricted spectrum of point mutations was found in the beta-tubulin gene from field strains than from laboratory-induced mutants. Five mutations in two codons were found in the beta-tubulin genes of 24 benomyl-resistant field strains (Table 1) versus 14 mutations in nine codons in the beta-tubulin genes of 14 laboratory-induced mutants (Table 2) (6,12,13,25,34).

DISCUSSION

In previous studies involving *V. inaequalis* and *V. pirina*, strains were classified as S, LR, MR, HR, or VHR according to their differential growth response on PDA and diethofencarb (10,15,28–30,33). Among 24 benomyl-resistant field strains, all except three with low resistance had mutations in codon 198 or codon 200 of the beta-tubulin gene. The results of this study indicate that a change in codon 198 or codon 200 of the beta-tubulin of *V. inaequalis* and *V. pirina* and of several other plant pathogens confers resistance to benomyl and implies a key role of the amino acids at these positions in the action of benzimidazole fungicides. In addition, each phenotypic class of *V. inaequalis*, with the exception of the LR class, was associated with a unique amino acid substitution at position 198 or 200. The same association between unique amino acid substitutions and benomyl resistance levels was observed in field strains of *V. pirina, M. fructicola, S. homoeocarpa*, and six species of *Penicillium*.

Results from Southern analysis performed at moderate stringency suggested that *V. inaequalis* contains only one copy of the beta-tubulin gene. This result, in conjunction with previous genetic data suggesting that only one benomyl-resistance gene exists in *V. inaequalis* (28,33), supports the assumption that the observed mutations in codons 198 and 200 are directly responsible for benomyl resistance in this plant pathogen. We did not investigate whether additional beta-tubulin sequences were present in the other fungi by Southern analysis with their homologous probes.

Our data were consistent with the hypothesis that certain mutations in the beta-tubulin gene confer sensitivity to diethofencarb as well as resistance to benomyl (6,10,29). Only those mutations in codon 198, converting the codon for glutamic acid in sensitive strains to alanine in resistant strains of six fungal species with the VHR phenotype or to glycine in one MR− strain of *V. inaequalis*, were associated with a change in phenotype from insensitivity to sensitivity to diethofencarb. Other substitutions in codon 198 did not automatically confer sensitivity to all *N*-phenylcarbamate fungicides. HR strains, with mutations changing codon 198 for glutamic acid to codons for lysine or valine, exhibited sensitivity to MDPC but insensitivity to diethofencarb. In addition, mutations in codon 200 from a phenylalanine codon to tyrosine codon were never associated with an altered sensitivity to diethofencarb. In transformation experiments with *N. crassa*, a mutation of codon 198 from a glutamic acid codon to a glycine codon conferred resistance to benomyl and sensitivity to diethofencarb (6). This mutation was also found in benomyl-resistant and diethofencarb-sensitive strain I-26 of *V. inaequalis* (Table 1).

The characterization of laboratory-induced mutants of model organisms such as *N. crassa* and *A. nidulans* has been very important for establishing the mode of action of the benzimidazole fungicides (3,12,13,25). However, their value in predicting whether a particular mutation will become a problem in the field remains unknown. The mutations in the beta-tubulin gene of benomyl-resistant field strains of several plant pathogenic fungi were limited to two codon positions (Tables 1 and 2). A possible reason for the reduced variation in mutations found among field strains as compared to laboratory-induced mutants is that mutations in other codons might interfere with the fitness of mutants and impose a selective disadvantage on these mutants under field conditions. Benomyl-resistant strains of *A. nidulans* with mutations in codons 50, 134, or 257 were also temperature-sensitive (B. R. Oakley, *personal communication*) (Table 2), and such strains would be expected to have a reduced fitness. Results from greenhouse and field studies indicate that many benomyl-resistant field strains exhibited a high level of fitness as evidenced by their persistence in populations (18,22). Some field strains of *V. inaequalis* collected from an orchard in Michigan more than 10 yr after benomyl application was discontinued contained a muta-

TABLE 2. Deduced amino acid substitutions in the beta-tubulins of laboratory-induced mutants with resistance to benzimidazole fungicides

Organism	Amino acid		Source or reference
	Substitution	Position	
Aspergillus nidulans	His to Tyr	6	13
	His to Leu	6[a]	12
	Tyr to Asn	50[b]	B. R. Oakley
	Tyr to Ser	50[b]	B. R. Oakley
	Gln to Lys	134[b]	B. R. Oakley
	Ala to Val	165[c]	12
	Glu to Asp	198	13
	Glu to Gln	198	13
	Glu to Lys	198[d]	13
	Phe to Tyr	200[d]	13
	Met to Leu	257[b]	B. R. Oakley
Neurospora crassa	Phe to Tyr	167	25
	Glu to Gly	198[d]	6
Saccharomyces cerevisiae	Arg to His	241	34

[a] Substitution confers resistance to benomyl and carbendazim, and tubulin of mutant exhibited decreased affinity for carbendazim (2).
[b] Point mutations found in heat-sensitive mutants (B. R. Oakley, *personal communication*).
[c] Substitution confers resistance to thiabendazole and supersensitivity to benomyl and carbendazim, and tubulin of mutant exhibited increased affinity for carbendazim (2).
[d] Mutations found in both laboratory-induced mutants and field strains.

tion in codon 198 from glutamic acid to alanine and exhibited very high resistance to benomyl in laboratory tests (17). However, a comparison of the deduced amino acid sequences from beta-tubulin genes of many organisms revealed a highly conserved glutamic acid in position 198, suggesting that there is considerable constraint on this residue (13). The observation that mutants with nonconservative amino acid substitutions at position 198 (from glutamic acid to lysine, glycine, or alanine) are common in the field was, therefore, surprising. The appearance of field mutants with a substitution of phenylalanine by tyrosine in position 200 was less surprising. A comparison of the amino acids in position 200 showed that considerable variation exists among different organisms and suggests a reduced constraint on this position. Provided that mutations in positions other than 198 or 200 of the beta-tubulin gene interfere with the fitness of mutants, it will be important to use field mutations in the genetic engineering of strains of fungi that are used in biocontrol (26).

Differential affinity of fungal tubulin to both *N*-phenylcarbamates and carbendazim, the toxic principle of benomyl, is likely the basis for the variation in resistance among the benomyl-resistant strains examined in this study. Reduced binding of carbendazim by crude extracts of tubulin was reported for laboratory-induced strains of *A. nidulans* (3), field strains of *V. nashicola* (8), and thiabendazole-resistant strains of the nematode *Haemonchus contortus* (19). The beta-tubulin genes of the *A. nidulans* strains used in binding studies by Davidse (3) contained unique codon substitutions that conferred resistance or supersensitivity to benomyl (12). In benomyl-resistant strain BEN17, the *Ben*A19 allele had a leucine codon in place of a histidine codon at position 6, and tubulin from this strain exhibited reduced affinity for carbendazim. Replacement of an alanine codon with a valine codon at position 165 in the *Ben*A16 allele of strain BEN14 was associated with both supersensitivity to benomyl and increased affinity of tubulin for carbendazim. Evidence implicating tubulin in the mode of action of *N*-phenylcarbamates was that germinating spores of VHR strains exposed to diethofencarb, MDPC, or *N*-phenylformamidoximes exhibited distortion of germ tubes identical to that observed when benomyl-sensitive strains were exposed to carbendazim (16,24). In addition, crude protein extracts from strains of *V. nashicola* with different levels of resistance to benomyl exhibited differential binding of *N*-phenylformamidoximes (9). The beta-tubulin genes of the mutants were not characterized.

Such results indicate that amino acids in positions 198 and 200 in beta-tubulin may play a critical, important role in the binding of *N*-phenylcarbamates and carbendazim. Substitution of glutamic acid at position 198 by glycine or alanine in benomyl-resistant strains could dramatically increase the affinity for both diethofencarb and MDPC, while substitution of glutamic acid by lysine or valine could increase the affinity for MDPC but not for diethofencarb. A similar model for differential binding of carbendazim and diethofencarb to beta-tubulin of benomyl-sensitive and benomyl-resistant strains was proposed by Fujimura et al (6). Structural analysis of carbendazim, *N*-phenylcarbamates, and *N*-phenylformamidoximes compounds with Alchemy II software (Tripos Associates, St. Louis, MO) revealed nearly superimposable shapes for the molecules (Koenraadt, *unpublished*). However, structural analysis of the beta-tubulin protein is necessary to substantiate the hypothesis that the *N*-phenylcarbamates fungicides bind to the altered carbendazim binding site, but such a study has not been possible, because of the intractable problem at crystallizing tubulin for X-ray diffraction studies (1).

The molecular basis for benomyl resistance in strains with the LR phenotype was not elucidated. Classic genetic analysis provided evidence that this trait was heritable and controlled by the same gene that conferred MR, HR, and VHR phenotypes to benomyl in *V. inaequalis* and *V. pirina* (28,30,33). Only a portion of the beta-tubulin gene of LR strain MINNS 118 was sequenced, and a mutation might be present in a portion of the gene that was not sequenced. It may also be possible that low resistance to benomyl was due to altered expression of the beta-tubulin gene. Characterization of a laboratory-induced benomyl-resistant strain of *Epichloe typhina* revealed a rearrangement in the 5′-flanking region of the beta-tubulin gene, and no alternations in the deduced amino acid sequence were found (2). Sequence variation in the 5′-flanking region was observed in LR strain MINNS 118 (Koenraadt, *unpublished*), but whether this variation altered the expression of the beta-tubulin gene and was responsible for the LR phenotype was not investigated.

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