Resistance to Benomyl Conferred by Mutations in Codon 198 or 200 of the Beta-Tubulin Gene of *Neurospora crassa* and Sensitivity to Diethofencarb Conferred by Codon 198

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ABSTRACT

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In previous work, we established that point mutations in codon 200 ($200^{\text{Phe to Tyr}}$) or 198 ($198^{\text{Glu to Ala}}$) of the beta-tubulin gene were associated with medium and very high resistance to benomyl in field strains of several plant-pathogenic fungi. Site-directed mutagenesis was performed to introduce identical mutations in codon 198 or 200 of a beta-tubulin gene from *Neurospora crassa.* At position 198 a codon for glutamic acid was altered to a codon for alanine. Alternatively, at position 200 a codon for phenylalanine was altered to a codon for tyrosine. Transformants were obtained that exhibited resistance to benomyl after transformation with plasmid constructs. Southern analysis confirmed that additional copies of beta-tubulin DNA were present in the transformants. Transformants with beta-tubulin DNA that were integrated with the codon for alanine also exhibited sensitivity to diethofencarb. Therefore, we conclude that mutations in codon 198 or 200 of the beta-tubulin gene confer resistance to benomyl in several benomyl-resistant plant-pathogenic fungi, as demonstrated in this study for *N. crassa,* and that an alanine codon at position 198 is associated with sensitivity to diethofencarb.

Control of apple scab, caused by *Venturia inaequalis* (Cooke) G. Wint., has been compromised by the development of resistance in the pathogen to the fungicide benomyl. Classic genetic analysis showed that resistance to benomyl in field strains of *V. inaequalis* and *V. pirina* Aderhold was caused by mutations in a single gene and that alleles of this gene conferred differing levels of resistance (11,16,18,20). In addition, genetic analysis suggested that the allele for very high resistance to benomyl also conferred sensitivity to the N-phenylcarbamate fungicide diethofencarb (8,17).

In *Aspergillus nidulans* (Eidam) G. Wint. and *Neurospora crassa* Shear et Dodge, molecular analysis of the beta-tubulin gene and transformation experiments demonstrated that certain point mutations in the beta-tubulin gene of these fungi were responsible for resistance to benomyl (6,9,10,15). Nucleotide sequence analysis also revealed point mutations in the beta-tubulin gene of benomyl-resistant strains of *V. inaequalis.* Codon 198 for glutamic acid in a sensitive strain was altered to lysine in a strain with high resistance or to alanine in a strain with very high resistance (VHR), and codon 200 for phenylalanine was altered to tyrosine in a strain with medium resistance (MR) to benomyl (13). Allele-specific oligonucleotide analysis showed that mutations in codon 198 or 200 were present in 20 additional benomyl-resistant strains of *V. inaequalis* that originated from different geographic regions (12). In addition, codon mutations identical to those in *V. inaequalis* were also associated with each benomyl-resistance level in strains of several other plant-pathogenic fungi (13).

The objective of this study was to establish that the mutations found in codons 198 and 200 of field strains of *V. inaequalis* were responsible for resistance to benomyl. Site-directed mutagenesis was performed to create the mutations in codons 198 or 200 of the beta-tubulin gene of *N. crassa.* The altered beta-tubulin DNA was used in transformation experiments with

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N. crassa, and medium amended with benomyl was used for the selection of transformants.

MATERIALS AND METHODS

Fungal and bacterial strains and plasmids. *Escherichia coli* strain JM109 was used as a host for cloning experiments and for the production of single-stranded DNA. *E. coli* strain BMH71-18 *mutS* (Promega, Madison, WI) was used as a host in transformations with plasmid DNA from the site-directed mutagenesis reactions. Plasmid pBT6, containing the entire beta-tubulin gene from *N. crassa,* was used as the source for the beta-tubulin gene (15). Plasmid pSELECT-1 (Promega) was used as the mutagenesis vector.

Strain *arg-3*(30300) A (FGSC 1086) of *N. crassa,* obtained through the Fungal Genetics Stock Center, Department of Microbiology, University of Kansas Medical Center, Kansas City, was used as a recipient strain in transformation experiments. The strain was grown in Vogel's minimal medium (21) supplemented with 15 g of sucrose and 20 mg of L-arginine per liter.

Construction of plasmid pTYR. Plasmid pBT6 was digested to completion with *Hin*dIII, and a 3.1-kb DNA fragment was separated from the vector DNA by agarose gel electrophoresis (14). The 3.1-kb DNA fragment was digested to completion with *Sal*I to give a 2.6-kb *Sal*I-*Hin*dIII DNA fragment that contained the entire beta-tubulin gene from *N. crassa.* Plasmid pSELECT-1 was digested to completion with *Hin*dIII and *Sal*I, and the 2.6-kb *Sal*I-*Hin*dIII fragment was cloned into pSELECT-1 to form plasmid pTYR.

Site-directed mutagenesis in the beta-tubulin gene. The beta-tubulin DNA from pBT6 contained a mutation in codon 167 that conferred resistance to benomyl (15). Primer A, a 18-mer oligonucleotide (5′-GGCTACCTTCTCCGTCGT) designed to alter codon 167 for tyrosine to a codon for phenylalanine, was identical in sequence to codons 165–169 of the beta-tubulin gene of benomyl-sensitive strains of *N. crassa.* Primer B, a 26-mer oligonucleotide (5′-GAGAACTCTGACGAGACATTCTGCAT), was designed to alter nucleotides in the third position of codons 196 and 199 of the beta-tubulin gene of *N. crassa* (Table 1). Primer B was identical in sequence to codons 194–201 of the beta-tubulin gene of benomyl-sensitive strains of *V. inaequalis.* Primer ASO^{MR} (5′-CGAGACATACTGCATTGA), used to alter codon 200 for phenylalanine to a codon for tyrosine, was identical in sequence to codons 198–202 of the beta-tubulin gene of strains of *V. inaequalis* with medium resistance to benomyl. Primer ASO^{VHR} (5′-CTCTGACGCGACATTCTG), used to alter codon 198 for glutamic acid to a codon for alanine, was identical in sequence to codons 196–200 of the beta-tubulin gene of field strains of *V. inaequalis* with very high resistance to benomyl. Primers ASO^{MR} and ASO^{VHR} were used as allele-specific oligonucleotides in a previous study (12). All the primers were synthesized in the Macromolecular Structure Facility, Department of Biochemistry, Michigan State University, East Lansing. Oligonucleotides were phosphorylated with T4 polynucleotide kinase as recommended by the manufacturer (Promega). An ampicillin repair oligonucleotide (5′-GTTGCCATTGCTTGCAGGCATCGTGGTG) was used in the mutagenesis reactions to repair the ampicillin resistance gene of recombinant pSELECT constructs.

Site-directed mutagenesis was performed with the Altered Sites system (Promega) as recommended by the manufacturer. Single-stranded DNA of pTYR was produced by infection of *E. coli* JM109 with helper phage R408 and then purified according to the Altered Sites protocol. The ampicillin repair nucleotide and primers A and B were annealed to the single-stranded DNA. Mutant strand synthesis and ligations were performed with T4 DNA polymerase and T4 DNA ligase. Products of the mutagenesis reaction were used to transform competent *E. coli* BMH71-18 *mutS* cells. The transformed cells were grown in Luria-Bertani (LB) medium (14) amended with 125 μg/ml of ampicillin to select transformants that contained a restored ampicillin resistance gene. Plasmid DNA from ampicillin-resistant transformants, extracted with a plasmid minipreparation procedure as recommended by the manufacturer, was used for transformation of competent *E. coli* JM109 cells. Single-strand plasmid DNA of ampicillin-resistant JM109 colonies was characterized by sequence analysis using Sequenase (United States Biochemical, Cleveland, OH) as recommended by the manufacturer to characterize codons 167 and 195–200. Plasmid pTYR, in which codons 167, 196, and 199 were altered, was designated pPHEVi (Table 1).

To alter codon 198 or codon 200, the 2.6-kb *Sal*I-*Hin*dIII DNA fragment of pPHEVi was cloned into pSELECT-1 to form $pPHEViAmp^s$ (Table 1). Mutagenesis with primers ASO^{MR} and ASO^{VHR} was performed as described above. Sequence analysis was used to verify the presence of altered codons. Plasmids $pPHEViAmp^s$, in which codon 200 was replaced by a codon for tyrosine or codon 198 was replaced by a codon for alanine, were designated as $pPHE^{MR}$ and $pPHE^{VHR}$, respectively (Table 1).

Transformation of *N. crassa.* Transformation of *N. crassa* was performed by the procedure of Vollmer and Yanofsky (22). Conidia (2×10^9) were germinated in flasks with 100 ml of Vogel medium with sucrose (15 g/L) and L-arginine (50 mg/L) at 30 C for 4–6 h in a shaker (200 rpm). The germinated conidia were collected by centrifugation, washed, and resuspended in 10 ml of 1 M sorbitol. Novozym 234 (50 mg) (Novo Industries, Copenhagen, Denmark) was suspended in 10 ml of 1 M sorbitol, filter-sterilized, and added to the conidial suspension. The suspension was shaken at 75 rpm for 1 h at 30 C, and spheroplasts were washed twice in 1 M sorbitol and once in STC (1 M sorbitol, 50 mM Tris HCl, pH 8.0, 50 mM $CaCl_2$). The spheroplasts were resuspended in 16 ml of STC, and then 4 ml of 40% PEG 4000, 50 mM of Tris HCl, pH 8.0, 50 mM of $CaCl_2$, and 200 μl of dimethyl sulfoxide were added.

For the transformation, 5 μl of STC with 1 μg of plasmid DNA was mixed with 2 μl of 50 mM spermidine·HCl; 5 μl of heparin solution (5 mg/ml STC) and 100 μl of spheroplasts were added; and the mixture was incubated on ice for 30 min. Then 1 ml of 40% PEG 4000, 50 mM of Tris HCl, pH 8.0, and 50 mM of $CaCl_2$ were added, followed by incubation for 20 min

TABLE 1. Plasmid designations and codon alternations in the beta-tubulin gene of *Neurospora crassa*

Plasmid[a]	Codon position[b]					Genotype[c]	Source or reference
	167	196	198	199	200		
pSELECT-1	...	...	...	...	...	Amp^s	Promega
pBT6	TAC-Y	TCC-S	GAG-E	ACC-T	TTC-F	Amp^r, Ben^r	15
pTYR	TAC-Y	TCC-S	GAG-E	ACC-T	TTC-F	Amp^s, Ben^r	This study
pPHEVi	TTC-F	TCT-S	GAG-E	ACA-T	TTC-F	Amp^r, Ben^s	This study
$pPHEViAmp^s$	TTC-F	TCT-S	GAG-E	ACA-T	TTC-F	Amp^s, Ben^s	This study
$pPHE^{MR}$	TTC-F	TCT-S	GAG-E	ACA-T	TAC-Y	Amp^r, Ben^r	This study
$pPHE^{VHR}$	TTC-F	TCT-S	GCG-A	ACA-T	TTC-F	Amp^r, Ben^r	This study

[a]Plasmids pPHEVi, $pPHE^{MR}$, and $pPHE^{VHR}$ are products of site-directed mutagenesis reactions. (MR = medium resistance; VHR = very high resistance).

[b]Single-letter abbreviations for amino acids: Y = tyrosine; F = phenylalanine; S = serine; E = glutamic acid; A = alanine; and T = threonine.

[c]Plasmids contain a defective (Amp^s) or repaired and functional (Amp^r) ampicillin resistance gene and a wild-type beta-tubulin gene (Ben^s) or a beta-tubulin gene with codon substitutions that confer resistance to benomyl (Ben^r).

at 20 C. The spheroplasts were mixed with 15 ml of regeneration top-agar (22) and then plated on 25 ml of selective medium with benomyl (0.5 mg/L) (Benlate 50% WP, E.I. duPont de Nemours and Co., Wilmington, DE).

Molecular characterization of transformants by Southern analysis. Colonies of *N. crassa* that grew on media with benomyl were individually transferred to fresh media. Mycelium from the edge of each putative transformant was then transferred to flasks with 100 ml of Vogel medium and grown for 2 days at room temperature on a rotary shaker at 100 rpm. The mycelium was harvested, and total DNA was extracted as previously described (13). Southern analysis (19) was performed to determine the mode of integration and the number of copies of the beta-tubulin gene in each transformant. Genomic DNA of transformants was digested to completion with the restriction enzymes *Xba*I and *Sma*I, separated by electrophoresis in a 0.5% agarose gel, and then transferred from the agarose gel to Gene-Screen Plus membrane as recommended by the manufacturer (Du Pont, Boston, MA). A 2.6-kb *Sal*I-*Hin*dIII beta-tubulin DNA fragment from pBT6 was labeled with alpha^{32}-dCTP using the random primed labeling method (3) and then used as a probe to detect homologous DNA sequences.

Growth response of transformants to fungicides. The reaction of transformants to benomyl, methyl-N-(3,5-dichlorophenyl) carbamate (MDPC), diethofencarb, technical grade (both from Sumitomo Chemical Co., Tukatsukasa, Takarazuka, Hyogo, Japan), or a combination of benomyl and diethofencarb was determined by transferring small pieces of mycelium to petri dishes containing Vogel's medium amended with 2.5 mg/L of each fungicide. The mycelial growth of transformants was determined after incubating the petri dishes at 30 C for 1–2 days.

RESULTS

Site-directed mutagenesis in the beta-tubulin gene. Sequence analysis of transformants obtained in the first set of mutagenesis reactions confirmed that codon 167 (TAC) for tyrosine in pTYR was altered to a codon for phenylalanine (TTC) in pPHEVi. In addition, codons 196 (TCC) and 199 (ACC) in pTYR were replaced by alternate codons (TCT) and (ACA) for serine and threonine, respectively. In the second set of mutagenesis reactions, codon 198 for glutamic acid (GAG) in pPHEViAmp^s was altered to a codon for alanine (GCG) in pPHE^{VHR}. Codon 200 for phenylalanine (TTC) in pPHEViAmp^s was altered to a codon for tyrosine (TAC) in pPHE^{MR}.

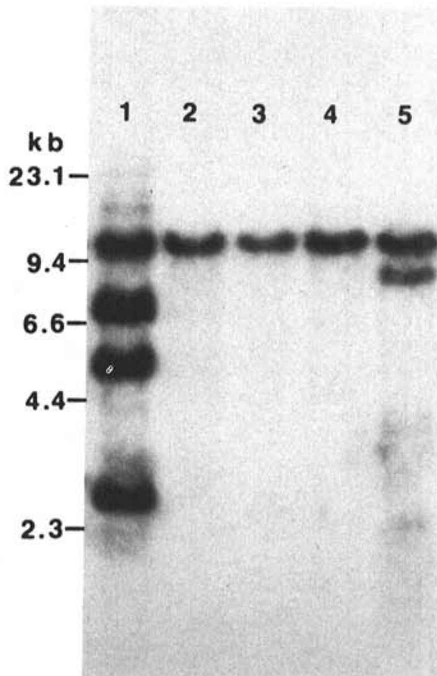

Fig. 1. Southern blot of DNA from parent strain *arg-3* (30300) A and putative transformants of *Neurospora crassa. Xba*I-*Sma*I digested genomic DNA was probed with a 2.6-kb beta-tubulin DNA fragment from *N. crassa.* Lane 1, transformant with multiple copies of pPHE^{VHR} (VHR = very high resistance); lane 2, parent strain *arg-3* (30300) A; lanes 3 and 4, nontransformants (false positives) without copies of pPHE^{VHR}; and lane 5, transformant with copy of pBT6 (control transformation). The endogenous beta-tubulin gene of *N. crassa* resides on a *Xba*I-*Sma*I DNA fragment with an estimated size of 10 kb.

Transformation of *N. crassa*. No fungal growth was observed when benomyl-sensitive spheroplasts were transformed with pPHEVi and plated on medium amended with benomyl. When benomyl-sensitive spheroplasts were transformed with pPHE^{MR} or pPHE^{VHR}, several fungal colonies developed on medium amended with benomyl.

Southern analysis of DNA from the nontransformed recipient strain showed that the beta-tubulin DNA probe hybridized with a 10-kb *Xba*I-*Sma*I DNA fragment. However, when DNA from benomyl-resistant transformants was subjected to Southern analysis, the probe hybridized with a 10-kb *Xba*I-*Sma*I DNA fragment and with several additional DNA fragments of variable size (Fig. 1, lane 1).

Growth of transformants on medium amended with fungicides. Transformants in which pPHE^{MR} or pPHE^{VHR} was integrated in the genome exhibited resistance to benomyl (Fig. 2). Those in which pPHE^{VHR} DNA was present exhibited reduced growth on medium amended with diethofencarb and exhibited no growth on medium amended with both benomyl and diethofencarb; the nontransformed parent strain *arg-3* (3030) A and transformants with pPHE^{MR} were not inhibited in growth (Fig. 2). In addition, plating of putative false transformants with pPHE^{VHR} that did not contain additional copies of the beta-tubulin gene (Fig. 1, lanes 3 and 4) grew freely on medium amended with diethofencarb (not shown). Sensitivity to MDPC was observed only in transformants with copies of pPHE^{VHR}, and growth of these transformants was completely arrested on medium amended with both benomyl and MDPC (not shown). Transformants with copies of pPHE^{MR} were insensitive to both diethofencarb (Fig. 2) and MDPC.

DISCUSSION

Classic genetic analysis (8,20), nucleotide sequence analysis (13), and allele-specific oligonucleotide analysis (12) provided circum-

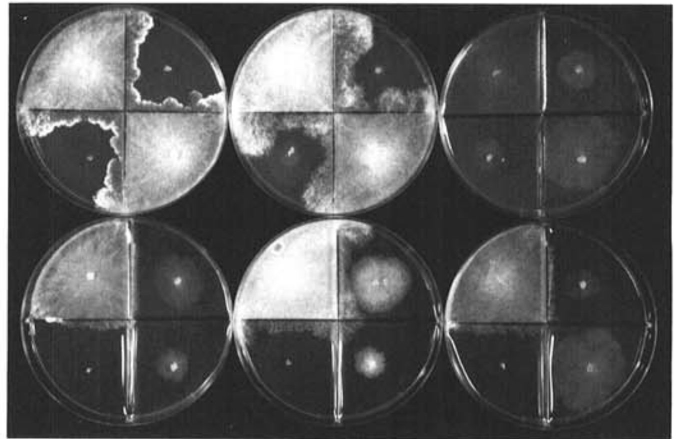

Fig. 2. Growth response after 1 day at 30 C of parent strain *arg-3* (30300) A and benomyl-resistant transformants of *Neurospora crassa* on Vogel's medium (upper left quadrant of each plate), Vogel's medium amended with benomyl (upper right quadrant), diethofencarb (lower right quadrant), and benomyl + diethofencarb (lower left quadrant). The petri dish in the upper right position contains the parent strain; the middle and left petri dishes contain transformants with pPHE^{MR} DNA (MR = medium resistance). The petri dishes in the lower row contain transformants with pPHE^{VHR} DNA (VHR = very high resistance). DNA extracted from mycelium of the strain in the center plate in the lower row contained several additional beta-tubulin genes, as shown by Southern blotting (Fig. 1, lane 1).

stantial evidence that mutations in codons 198 and 200 of the beta-tubulin gene were responsible for field resistance to benomyl in several plant-pathogenic fungi. The studies also suggested that sensitivity to the N-phenylcarbamate fungicides diethofencarb and MDPC was caused by mutations in codon 198 of the beta-tubulin gene. Here, based on transformation experiments with *N. crassa*, we provide direct evidence that mutations in codons 198 and 200 are responsible for resistance to benomyl. In addition, conclusive evidence is provided that a mutation for alanine in codon 198 causes sensitivity to diethofencarb and MDPC.

The results of Southern analysis showed that additional beta-tubulin DNA copies were present in each benomyl-resistant transformant. A 10-kb DNA fragment, containing the endogenous beta-tubulin gene of *N. crassa*, hybridized with the beta-tubulin DNA probe in all DNA samples. Additional DNA fragments that hybridized with the probe were observed only in DNA samples from transformants. Because the restriction enzymes *Sma*I and *Xba*I have no sites in the structural beta-gene of *N. crassa*, we concluded that each additional band resulted from a random integration of the plasmid with the beta-tubulin gene with mutations in codon 198 or 200. The exhibition of benomyl resistance of transformants plated on medium amended with benomyl was in agreement with the hypothesis that mutations in codons 198 and 200 were responsible for resistance to benomyl (12,13). Also, characterization of the beta-tubulin gene from benomyl-resistant mutants of *A. nidulans* and *N. crassa* revealed mutations in codons 198 and 200 (4,6,10). In addition, the observed phenotypes of transformants plated on fungicide-amended media supported the hypothesis that substitution of alanine for glutamic acid at position 198 in beta-tubulin was responsible for increased sensitivity to diethofencarb and MDPC. For example, only transformants with copies of $pPHE^{VHR}$ DNA exhibited significantly retarded growth on medium amended with diethofencarb, and their growth was completely arrested on medium amended with a combination of benomyl and diethofencarb. A similar growth response was observed for transformants of *N. crassa* in which beta-tubulin DNA was introduced with a mutation of codon 198 for glycine (6). Recently, transformation experiments with *N. crassa* showed also that $198^{Glu\ to\ Lys}$ conferred resistance to benomyl (4).

The phenotypes of transformants with $pPHE^{VHR}$ DNA of *N. crassa* and of VHR strains of plant-pathogenic fungi are not identical. The growth of transformed isolates of *N. crassa* on medium amended with diethofencarb is significantly reduced, while growth of VHR strains of plant-pathogenic fungi is completely arrested. This apparent discrepancy is explained by the presence of two beta-tubulin isotypes in transformants of *N. crassa*. Expression of the endogenous beta-tubulin gene in the transformants will give rise to a beta-tubulin isotype that is sensitive to benomyl but insensitive to diethofencarb. Expression of the $pPHE^{VHR}$ copies will give rise to a beta-tubulin isotype that is resistant to benomyl but sensitive to diethofencarb.

Plasmid $pPHE^{VHR}$ or derivatives of this plasmid might be useful in fungal transformation experiments. Because of their sensitivity to diethofencarb, real transformants can be distinguished from false positives. For instance, the false positives (Fig. 1, lanes 3 and 4) grew freely on medium amended with diethofencarb, unlike transformants with $pPHE^{VHR}$ copies. In addition, transformation with $pPHE^{VHR}$ would allow a phenotypical discrimination between the events of gene replacement by homologous recombination and by random integration into the genome. In the event of gene replacement, transformants will express only the introduced beta-tubulin gene. The expression of the beta-tubulin isotype 198^{Ala} will confer benomyl resistance and diethofencarb sensitivity. In the event of random integration into the genome, transformants will have two beta-tubulin isotypes, because both the endogenous (198^{Glu}) and the introduced beta-tubulin gene (198^{Ala}) are expressed. Therefore, these transformants will exhibit growth on medium amended with diethofencarb, although the growth will be slightly retarded.

The point mutations that are present in $pPHE^{MR}$ and $pPHE^{VHR}$ are common among benomyl-resistant field strains of several plant-pathogenic fungi (13). The mutation $198^{Glu\ to\ Ala}$ does not appear to interfere with fitness, because field strains of *V. inaequalis* with the VHR phenotype were collected more than 10 yr after benomyl application was discontinued (13). A comparison of mutations in beta-tubulin DNA from laboratory-induced and field strains with resistance to benomyl revealed that point mutations in 11 different codons were present in laboratory-induced mutants, while point mutations in only codons 198 and 200 were observed in field strains (13). It was hypothesized that some of the codon changes that were exclusively observed in laboratory-induced mutants, including the alteration of codon 167 in the beta-tubulin gene of strain Bm1511 (1), might interfere with the biological activity of beta-tubulin and consequently affect the fitness of the mutant (13).

It is known that mutations in codons 6 and 165 of the beta-tubulin gene interfere with the binding of carbendazim to tubulin (2,9). The results of this study are in agreement with the hypothesis that certain mutations in codon 198 of the beta-tubulin gene decrease the affinity of tubulin for carbendazim and increase the affinity for N-phenylcarbamates (4–6,13). Binding studies with N-phenylcarbamates showed that the affinity of tubulin from benomyl-resistant strains of fungi increased for those compounds, but the mutations in these strains had yet to be characterized (7).

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