Mutations Leading to Substitutions at Amino Acids 198 and 200 of Beta-Tubulin that Correlate with Benomyl-Resistance Phenotypes of Field Strains of *Botrytis cinerea*

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ABSTRACT

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Three phenotypes were identified among benomyl-resistant strains of *Botrytis cinerea* in Israel when tested for sensitivity to carbendazim (MBC) and diethofencarb (NPC): $Ben^{HR}NPC^{S}$ = highly resistant to MBC (50% effective concentration [EC_{50}] > 50 μg/ml) and sensitive to 0.5 μg/ml NPC; $Ben^{MR}NPC^{R}$ = moderately resistant to MBC ($10 \leq EC_{50} < 20$ μg/ml) and resistant to 10 μg/ml NPC; and $Ben^{HR}NPC^{R}$ = highly resistant to MBC and resistant to NPC. A 1-kb fragment of the wild-type gene encoding for beta-tubulin (designated *benA*) in *B. cinerea* was cloned and sequenced. The deduced partial amino acid sequence of the *B. cinerea* beta-tubulin showed a high degree of similarity to beta-tubulins of other filamentous fungi. A polymerase chain reaction approach was used to amplify and sequence 992-bp *benA* fragments from strains representing the three phenotypes. In the eight Ben^{R} strains analyzed, three single base-pair mutations were identified and found to correlate with the different phenotypes: codon 198, encoding glutamic acid in the wild type, was changed to an alanine codon in the $Ben^{HR}NPC^{S}$ phenotype or to a lysine codon in the $Ben^{HR}NPC^{R}$ phenotype; codon 200, encoding phenylalanine, was changed to a tyrosine codon in the $Ben^{MR}NPC^{R}$ phenotype. These mutations were similar to those identified in benomyl-resistant field strains of other phytopathogenic fungi.

Additional keywords: *Botryotinia fuckeliana*, fungicide resistance, gray mold, negative cross-resistance, *N*-phenylcarbamate.

Gray mold, caused by *Botrytis cinerea* Pers.:Fr., is an important disease of a number of crops in Israel (19,38). The spread of *B. cinerea* strains resistant to benzimidazoles (Ben^{R}) and dicarboximides (Dic^{R}) greatly reduced the usefulness of these fungicides for chemical control of gray mold. Following the discovery that benomyl-resistant *B. cinerea* showed sensitivity to *N*-phenylcarbamate (NPC) compounds (25), the fungicidal mixture of carbendazim (MBC) plus diethofencarb was introduced in an attempt to exploit the "negative cross-resistance" phenomenon between NPCs (such as diethofencarb) and benzimidazoles (22,37) to control both Ben^{S} and Ben^{R} strains of *B. cinerea* and some other plant pathogens. Reports of Ben^{R} strains of *B. cinerea* from Europe (6,10,13,25,28), Asia (4,22), and Australia (6) indicated that they were all sensitive to NPCs, and promising results were obtained in trials to control mixed populations ($Ben^{S}NPC^{R}$ + $Ben^{R}NPC^{S}$) of *B. cinerea* on grapes (4,12,25) and protected crops (13,20) by the application of mixtures of benzimidazoles plus NPC. Genetic analyses of many $Ben^{R}NPC^{S}$ strains from different countries, including Israel, have shown that all of them carry allelic mutations at a single locus that also pleiotropically render them sensitive to NPC (6). However, in 1988, unsatisfactory gray mold control was reported from a cucumber plot in Israel experimentally treated with MBC + NPC. The insufficient fungicidal efficacy was attributed to the occurrence of *B. cinerea* strains resistant to both MBC and NPC ($Ben^{R}NPC^{R}$) (20). Similar strains were later found in other experimental plots in Israel treated with MBC + NPC. Some of these strains were also resistant to dicarboximides ($Ben^{R}NPC^{R}Dic^{R}$) (5). $Ben^{R}NPC^{R}$ strains have also been reported in France (24), Italy (7,31), and the United Kingdom (11). On the basis of studies of hyphal morphology, mitosis, and NPC metabolism in *B. cinerea,* Suzuki et al (37) suggested that NPCs interfere with the formation or function of microtubules in the $Ben^{R}NPC^{S}$ strains, as does MBC in wild-type strains. The presumed mutated locus was the structural gene of beta-tubulin, and the mutation was presumed to result in beta-tubulin with reduced affinity to MBC and higher affinity to NPCs. On the basis of this scheme, Katan et al (20) hypothesized that the $Ben^{R}NPC^{S}$ and $Ben^{R}NPC^{R}$ phenotypes of *B. cinerea* carry different allelic mutations, which are distinguishable by their pleiotropic effect on sensitivity to NPC.

The occurrence of field strains of *B. cinerea* with low, moderate, and high levels of resistance to MBC have been reported (6,7,11, 12,15,24,31). Some of these strains are also resistant to NPC, but variations in methodology and criteria of phenotype definition do not allow direct comparison of data on strains from different countries. Genetic analysis of $Ben^{R}NPC^{R}$ strains from Italy have shown that their Ben^{R} mutations are allelic to those in $Ben^{R}NPC^{S}$ strains, although they do not have a similar pleiotropic effect (7).

Bioactive benzimidazoles are specific inhibitors of microtubule assembly that act by binding to their heterodimeric subunit, the tubulin molecule (2). The binding affinity of benzimidazole to tubulin in vitro correlates with the sensitivity of strains to these fungicides (3). Sheir-Neiss et al (35) have demonstrated that *benA* is the structural gene for beta-tubulin in *Aspergillus nidulans* and that beta-tubulin mutations can be isolated as benomyl-resistance mutations. Cloning, sequence analyses, and transformation experiments with beta-tubulin genes from Ben^{S} and Ben^{R} strains of several fungal species have provided conclusive evidence for the linkage between beta-tubulin structure and benzimidazole sensitivity of fungi (8,18,23,26,29). The molecular basis for the mode of action and the linkage between mutations in the beta-tubulin gene and sensitivity to NPCs have recently been investigated in *Neurospora crassa* (8,9).

In this study, we identify mutations in the beta-tubulin gene that correlate with phenotypes of *B. cinerea* strains that differ in their sensitivity to benzimidazole and NPC fungicides.

MATERIALS AND METHODS

Fungicides. The following fungicides were used: carbendazim (MBC; Delsene 50 DF, E.I. DuPont de Nemours & Co., Wilmington, DE) and diethofencarb (25WP, Sumitomo Chemical Co., Takarazuka, Japan). To prepare fungicide-amended media, suspensions of fungicides in sterile deionized water were added to autoclaved potato-dextrose agar (PDA) at 45 C. Fungicide concentrations are expressed in micrograms per milliliter of active ingredient.

TABLE 1. Point mutations and deduced amino acid substitutions in the beta-tubulin gene of field strains of *Botrytis cinerea* exhibiting differential resistance to benzimidazole (Ben), *N*-phenylcarbamate (NPC), and dicarboximide (Dic) fungicides

Isolate	Host	Phenotype[a]			Codon substitution	Amino acid in position		
		Ben	NPC	Dic		198	199	200
GLX-553[b]	Gloxinia	S	R	S	None	Glu	Thr	Phe
VIT/HULD-8[b]	Grape	S	R	S	None	Glu	Thr	Phe
CU/87	Cucumber	HR	S	R	GAG to GCG	Ala	Thr	Phe
CU/91-88	Cucumber	HR	S	R	GAG to GCG	Ala	Thr	Phe
TUT/TIR-23	Strawberry	HR	S	R	GAG to GCG	Ala	Thr	Phe
CU/91-7	Cucumber	MR	R	R	TTC to TAC	Glu	Thr	Tyr
CU/SFA-17	Cucumber	MR	R	R	TTC to TAC	Glu	Thr	Tyr
FAR-7	Cucumber	HR	R	S	GAG to AAG	Lys	Thr	Phe
FAR-9	Cucumber	HR	R	S	GAG to AAG	Lys	Thr	Phe
FAR-11	Cucumber	HR	R	S	GAG to AAG	Lys	Thr	Phe

[a] S = sensitive; R = resistant; MR = moderately resistant; HR = highly resistant.
[b] Wild-type strains.

Strains of *B. cinerea*. Wild-type strains (Ben^SNPC^R) were from the laboratory collection at the Agricultural Research Organization, Bet Dagan, Israel. Ben^R strains were collected in surveys conducted in 1981 and 1986–1991 and characterized as either NPC^S or NPC^R (4,5,19,20). Cultures were maintained on slants of fungicide-free PDA. To determine conidial germination in the presence of MBC, PDA plates amended with MBC (0, 0.5, 5, 10, 20, or 50 μg/ml) were inoculated. Germination was examined by microscopic observation after a 20-h incubation period at 22 C. To compare mycelial growth rate, PDA plates with MBC (0, 0.5, 5, 10, 20, and 50 μg/ml) were centrally inoculated with mycelial plugs (4 mm in diameter) from the margins of 3-day-old colonies growing on PDA and incubated at 22 C. Colony diameter was measured after 3 days, and percentage of radial growth was calculated relative to growth on unamended PDA. The MBC concentration that inhibited radial growth by 50% (EC_{50}) was also determined. The reaction of the strains to NPC was confirmed by a germination test on PDA amended with diethofencarb (0.5 and 10 μg/ml).

Nucleic acid preparation, polymerase chain reaction (PCR), and nucleic acid analysis. Two wild-type and eight Ben^R field isolates of *B. cinerea* were used. The isolates, their hosts of origin, and their sensitivities to benzimidazole, NPC, and dicarboximide fungicides are listed in Table 1. Conidia were suspended in 80 ml of potato-dextrose broth in 250-ml flasks, and the cultures were incubated on a rotary shaker at 140–160 rpm for 3–4 days at 20–24 C. The mycelium was harvested by centrifugation, washed twice with sterile deionized water, frozen at −20 C, and lyophilized prior to extraction of nucleic acids. The dry mycelium was placed in a 15-ml polypropylene tube. To pulverize the mycelium, a spatula was inserted into the tube, and the the sample was vortexed for 2 min. The powder was suspended in 2 ml of lysis buffer (50 mM Tris-HCl, pH 8.0, 50 mM EDTA, 2% sodium dodecyl sulfate, 1% β-mercaptoethanol). RNase A (25 μg/ml) was added to the suspension. After an incubation period of 30 min (37 C), 100 μg/ml of proteinase K (Böehringer Mannheim, Mannheim, Germany) was added, and the mixture was incubated at 65 C for 1 h. The DNA was purified by two phenol-chloroform (1:1) extractions, a single chloroform extraction, and ethanol precipitation. The DNA pellet was resuspended in 0.5 ml of TE buffer (10 mM Tris-HCl, pH 7.4, 1 mM EDTA, pH 8.0), and aliquots were electrophoresed alongside lambda phage DNA in order to assess DNA quantity and quality. The DNA was used for PCR and Southern analysis.

To obtain a fragment of the *B. cinerea* beta-tubulin gene, a PCR approach was used. On the basis of conserved regions of beta-tubulin polypeptides deduced from several fungal beta-tubulin genes, degenerate oligonucleotide primers (Fig. 1) were synthesized. The primers were designed with restriction sites at the 5′ end to facilitate cloning of the PCR products. These primer mixtures corresponded to amino acids Trp101 to Thr106 (5′ primer) and Gln426 to Ser420 (3′ primer). The numbers following the amino acid designation indicate the location of the amino acid on the basis of several deduced beta-tubulin gene products

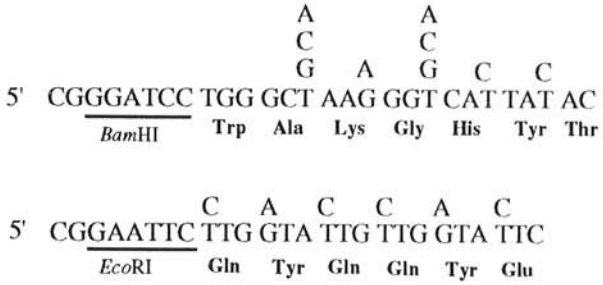

Fig. 1. Oligonucleotide mixtures used as primers for amplification of beta-tubulin-related fragments from genomic DNA. Polymerase chain reaction conditions are described in the text.

(23,29). In addition to being highly conserved, these primer sites were chosen to encompass the DNA encoding for amino acids 165, 167, 198–200, and 241, in which mutations have been reported that correlate with fungal resistance to benomyl (17,18,29). The degeneracy was complete to provide for any codon preference. PCRs were carried out in 100-μl reaction volumes on a thermal cycler (Ericomp, San Diego, CA). Cloned *Taq* polymerase (2.5 units, JV Magess-Bio, Moscow, Russia) was used in each reaction tube containing about 2 μg of genomic DNA as template in 25 mM Tris-HCl (pH 9.3), 50 mM KCl, 2 mM $MgCl_2$, 0.01% gelatin, 25 μg of activated thymus DNA, 200 μM of each dNTP, and 1 μg of each primer. A low-stringency program (1.5 min at 92 C followed by 35 cycles of 2 min at 50 C, 5 min at 60 C, and 1 min at 92 C) was used to allow for possible minor mismatches. The reaction products were analyzed by agarose gel electrophoresis (32) and subjected to endonuclease digestion when required. The plasmid Bluescript SK- (Stratagene, La Jolla, CA) was used for cloning experiments, and the cloned products were propagated in *Escherichia coli* strain DH5α.

Double-stranded DNA sequencing was performed by the dideoxy chain termination method (33) with the *Taq* DyeDeoxy Terminator Cycle Sequencing kit and the 373A DNA Sequencer (Applied Biosystems, Foster City, CA). Purified DNA (Magic Miniprep [Promega, Madison, WI] for plasmids and Jet Sorb [Genomed, Bad Deynhausen, Germany] for extraction of PCR products from agarose) was used as template.

Southern analysis of *B. cinerea* genomic DNA was performed at various stringency levels, according to Sambrook et al (32). Approximately 3 μg of genomic DNA was digested with the appropriate endonucleases, resolved on a 0.8% agarose gel, and transferred to a nylon membrane (Magna NT, MSI Inc., Westborough, MA). Hexamer-labeled pOY91 was used as a probe.

RESULTS

Characterization of strains. Wild-type isolates could not grow at 0.5 μg/ml of MBC but were resistant to 10 μg/ml of NPC, as reported previously (4). Phenotypes of Ben^R strains were

confirmed as either NPC^S (sensitive to 0.5 μg/ml of NPC) or NPC^R (resistant to 10 μg/ml of NPC) (20). Variation was observed in the germination and growth of Ben^R strains on MBC-amended media (Fig. 2; Table 2). Conidia of all Ben^R strains gave rise to normal germ tubes at 0.5–10 μg/ml of MBC (Fig. 2A, C, and E). However, at 50 μg/ml of MBC, some Ben^RNPC^R strains exhibited a sensitive reaction characterized by short, distorted germ tubes (Fig. 2D). The remaining Ben^RNPC^R strains and all Ben^RNPC^S strains examined had normal germ tubes at this MBC concentration (Fig. 2B and F); less pronounced inhibition occurred at 20 μg/ml of MBC.

The differences observed in conidial germination among Ben^R strains were paralleled by differences in mycelial growth on MBC-amended media (Table 2). All eight NPC^S strains were highly resistant to MBC, with $EC_{50} > 50$ μg/ml. Among the 12 NPC^R strains, two categories were evident with respect to the level of MBC resistance: 1) eight strains, whose germination was inhibited at 50 μg/ml (Fig. 2D), did not grow on media amended with 20 μg/ml or more of MBC, with $10 \leq EC_{50} < 20$ μg/ml; and 2) four strains exhibiting normal germination at 50 μg/ml of MBC (Fig. 2F) were also highly resistant to MBC in the hyphal growth test, with $EC_{50} > 50$ μg/ml. Thus, three phenotypes were identified among the 20 Ben^R strains examined (Table 2): $Ben^{HR}NPC^S$ = highly resistant to MBC and sensitive to NPC; $Ben^{MR}NPC^R$ = moderately resistant to MBC and resistant to NPC; and $Ben^{HR}NPC^R$ = highly resistant to MBC and resistant to NPC. Forty additional isolates from Israel were subjected to the rapid and simple conidial germination test, and the following results were obtained: Ben^S (wild-type) strains were always NPC^R; $Ben^{HR}NPC^S$ strains were the only Ben^R phenotypes found prior to 1988 or where NPC fungicides have not been used; $Ben^{HR}NPC^R$ strains were found only at one site, in 1988 (20); and $Ben^{MR}NPC^R$ strains appeared, along with $Ben^{HR}NPC^S$, in sites treated with MBC + NPC and sampled between 1989 and 1991 (5). Two or three representative isolates of each phenotype were used, along with two wild-type strains, to study the beta-tubulin gene (Table 1).

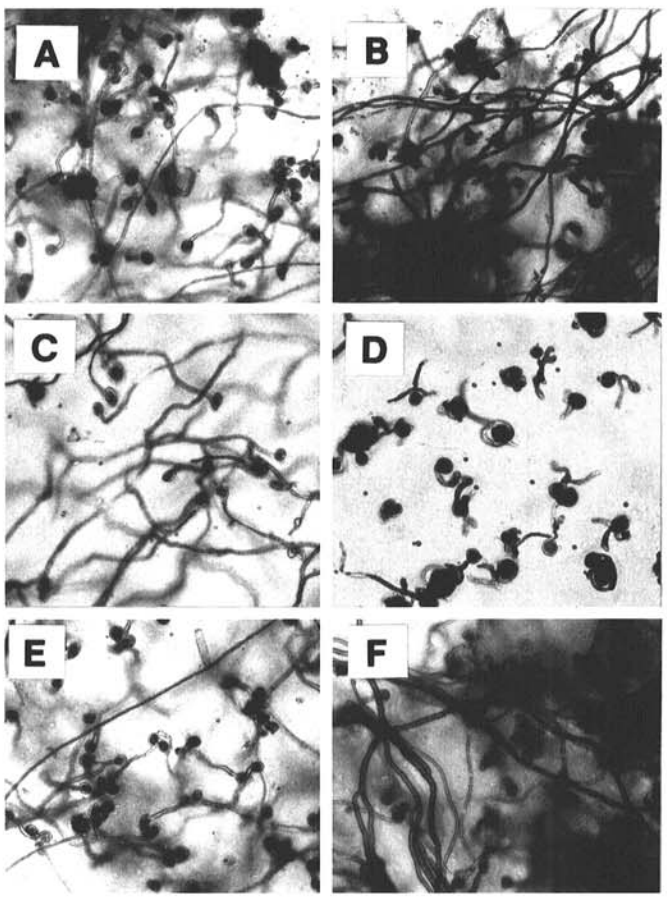

Fig. 2. Conidial germination of benomyl-resistant *Botrytis cinerea* on potato-dextrose agar amended with **A, C, and E,** 10 μg/ml or **B, D, and F,** 50 μg/ml of carbendazim (MBC). Phenotypes: **A and B,** $Ben^{HR}NPC^S$ = highly resistant to MBC and sensitive to 0.5 μg/ml of diethofencarb (NPC); **C and D,** $Ben^{MR}NPC^R$ = moderately resistant to MBC and resistant to 10 μg/ml of NPC; **E and F,** $Ben^{HR}NPC^R$ = highly resistant to MBC and resistant to NPC. Stained with cotton blue after 20 h incubation at 22 C.

Cloning and analysis of a 1-kb fragment of the wild-type beta-tubulin gene of *B. cinerea*. Initial analysis of PCR products by agarose gel electrophoresis provided evidence of a single amplified product approximately 1 kb in length. Endonuclease restriction digest analysis of the amplified product indicated the presence of a *Bam*HI site within the fragment. Subsequently, a 218-bp *Eco*RI-*Bam*HI fragment and a 774-bp *Bam*HI DNA fragment were cloned into the Bluescript vector. Both strands of the two cloned fragments, designated pOY90 and pOY91, respectively, were sequenced. The junction between the two fragments was sequenced with an appropriate oligonucleotide and the undigested PCR product as template. The nucleotide and deduced amino acid sequences are presented in Figure 3. The nucleotide sequence has been submitted to the GenEMBL database under accession number X73133. A tentative 56-bp intervening sequence was identified on the basis of the *N. crassa* consensus 5′ and 3′-splice junction sequences (14,29). The position of this tentative intron (Fig. 3) is identical (in conjunction with the coding region) to intron 6 in several fungal beta-tubulin genes (23,27,29,36). On the basis of the similarity of the deduced amino acid sequence of the *B. cinerea* beta-tubulin fragment to that of beta-tubulins in other fungal species (Fig. 4), we adopted the *A. nidulans* gene nomenclature convention and designated this gene *benA*. This is the first *B. cinerea* sequenced gene to be named, and its genotypic designation corresponds to the phenotypic one. The G+C content of the gene fragment is 48%, similar to the 51% G+C content found in the *A. nidulans benA* and *tubC* genes (27) and slightly lower than that of the *N. crassa* gene, which is 56% (29).

In at least three filamentous fungi, *A. nidulans* (27), *Colletotrichum graminicola* (30), and *C. gloeosporioides* (1), evidence has been provided for the presence of two beta-tubulin genes. We therefore attempted to determine whether a second, closely related beta-tubulin gene can be found in *B. cinerea*. Southern blot analysis of DNA from strains CU/87, GLX-553, and VIT/HULD-8 digested with *Bam*HI, *Eco*RI, *Hin*dIII, *Hpa*II, *Pst*I, and *Acc*65I revealed no evidence for the presence of additional DNA fragments similar to *benA* (data not shown). Thus, only single bands (excluding the analysis of *Pst*I-digested DNA, where the presence of two bands was expected) indicative of labeled-pOY91 hybridization were evident.

Molecular analysis of the beta-tubulin gene in Ben^R strains of *B. cinerea*. To determine whether point mutations in the region spanning amino acids 159–265 were linked to resistance to benzimidazole fungicides, we analyzed the nucleotide sequence encoding for this stretch of amino acids. Degenerate primers (Fig. 1) were used to obtain amplified fragments from genomic DNA extracted from eight field isolates exhibiting resistance to

TABLE 2. Radial growth (percentage of control) of benzimidazole fungicide-resistant strains of *Botrytis cinerea* on carbendazim (MBC)-amended media

	MBC (μg/ml)				
Phenotype[a]	5	10	20	50	EC_{50}[b]
$Ben^{HR}NPC^S$ (8)	96 ± 2[c]	93 ± 2	88 ± 2	79 ± 1	>50
$Ben^{MR}NPC^R$ (8)	79 ± 2	58 ± 2	tr[d]	tr	≥10, <20
$Ben^{HR}NPC^R$ (4)	95 ± 1	89 ± 2	81 ± 1	63 ± 1	>50

[a] Ben^{HR} and Ben^{MR} = highly and moderately resistant to MBC, respectively; NPC^S and NPC^R = sensitive and resistant to diethofencarb, respectively. Number of isolates is in parentheses.
[b] MBC concentration at which growth is reduced by 50%.
[c] Average ± standard error.
[d] Traces.

benzimidazole fungicides. Following gel purification, these fragments were used as templates for direct sequencing.

In the eight strains analyzed, resistance was correlated with single nucleotide substitutions, conferring changes in either amino acid 198 or 200 (Table 1). The nucleotide (and corresponding amino acid) changes observed were GAG (glutamic acid) to GCG (alanine) or AAG (lysine) at amino acid position 198 or TTC (phenylalanine) to TAC (tyrosine) at amino acid position 200. These changes correlated with the differential resistance to benzimidazole and NPC fungicides. Thus, codon 198, which encodes glutamic acid in the $Ben^{S}NPC^{R}$ (wild-type) strains, was substituted by lysine or alanine codons in the $Ben^{HR}NPC^{R}$ and $Ben^{HR}NPC^{S}$ strains, respectively. Codon 200, which encodes for phenylalanine in the $Ben^{S}NPC^{R}$ strains, was substituted by a tyrosine codon in the $Ben^{MR}NPC^{R}$ strains. The following allele designations were assigned to the *benA* mutations of *B. cinerea:* FY200 ($Ben^{MR}NPC^{R}$), EA198 ($Ben^{HR}NPC^{S}$), and EK198 ($Ben^{HR}NPC^{R}$). No differences between the nucleotide sequences encoding for other amino acids within the sequenced region were found among the strains analyzed; the GCT codon (for alanine) at amino acid position 165 and the CGT codon (for arginine) at position 241 were conserved in all strains analyzed.

DISCUSSION

We cloned and sequenced approximately two-thirds of the wild-type ($Ben^{S}NPC^{R}$) gene encoding for beta-tubulin in *B. cinerea.* The deduced amino acid sequence showed a high degree of similarity to those of other filamentous fungi (Fig. 4). Degrees of similarity ranged from 81% (*C. gloeosporioides* [1]) to 97% (*Venturia inaequalis* [23]). On the basis of the high degree of similarity observed, we designated this locus *benA*. Of the two *A. nidulans* beta-tubulin genes, the *B. cinerea benA* fragment most resembled the constitutively expressed *benA* gene. Similarly, the *B. cinerea benA* fragment shared a greater deduced amino acid identity with *tub-2* than with *tub-1* of *C. graminicola* (30). This information on *B. cinerea benA* could provide the basis for initial analysis of *B. cinerea* gene structure. Thus, even though this is a partial report, it is the first in which *B. cinerea* putative intron boundaries are presented.

Southern blot analyses of three different strains suggested that *B. cinerea* has only a single copy of *benA* and that highly similar DNA sequences (perhaps encoding for an additional beta-tubulin) are not present. In addition, no polymorphism in the *benA* sequences analyzed from the different strains was observed. The suggestion that no additional highly similar beta-tubulins are present in *B. cinerea* is supported by the fact that during the direct sequencing of PCR products primed with the degenerate oligonucleotide mixture no "overlay," indicative of template mixtures, was observed. Because the PCR and sequencing oligonucleotides used were designed on the basis of conserved regions of beta-tubulin common to both beta-tubulins (in species where two beta-tubulins have been found), it is assumed that if two similar beta-tubulin species (sharing sequence similarity to the oligonucleotide mixture) were present in the *B. cinerea* genome, at least two different DNA sequences would have been observed in the direct-sequencing reactions carried out on the amplified DNA fragments.

Sequence analysis of the PCR products, obtained following the amplification of a major portion of the beta-tubulin gene from 10 field strains of *B. cinerea,* correlated mutations leading to substitutions at amino acids 198 and 200 of beta-tubulin with various benomyl-resistant phenotypes. All the benomyl-resistant strains contained single base-pair mutations resulting in an amino acid substitution in the deduced beta-tubulin polypeptide (Table 1), and these substitutions correlated with differential resistance to benzimidazole and NPC fungicides. In a previous study (26), sequence analysis of beta-tubulin-encoding fragments from a wild-

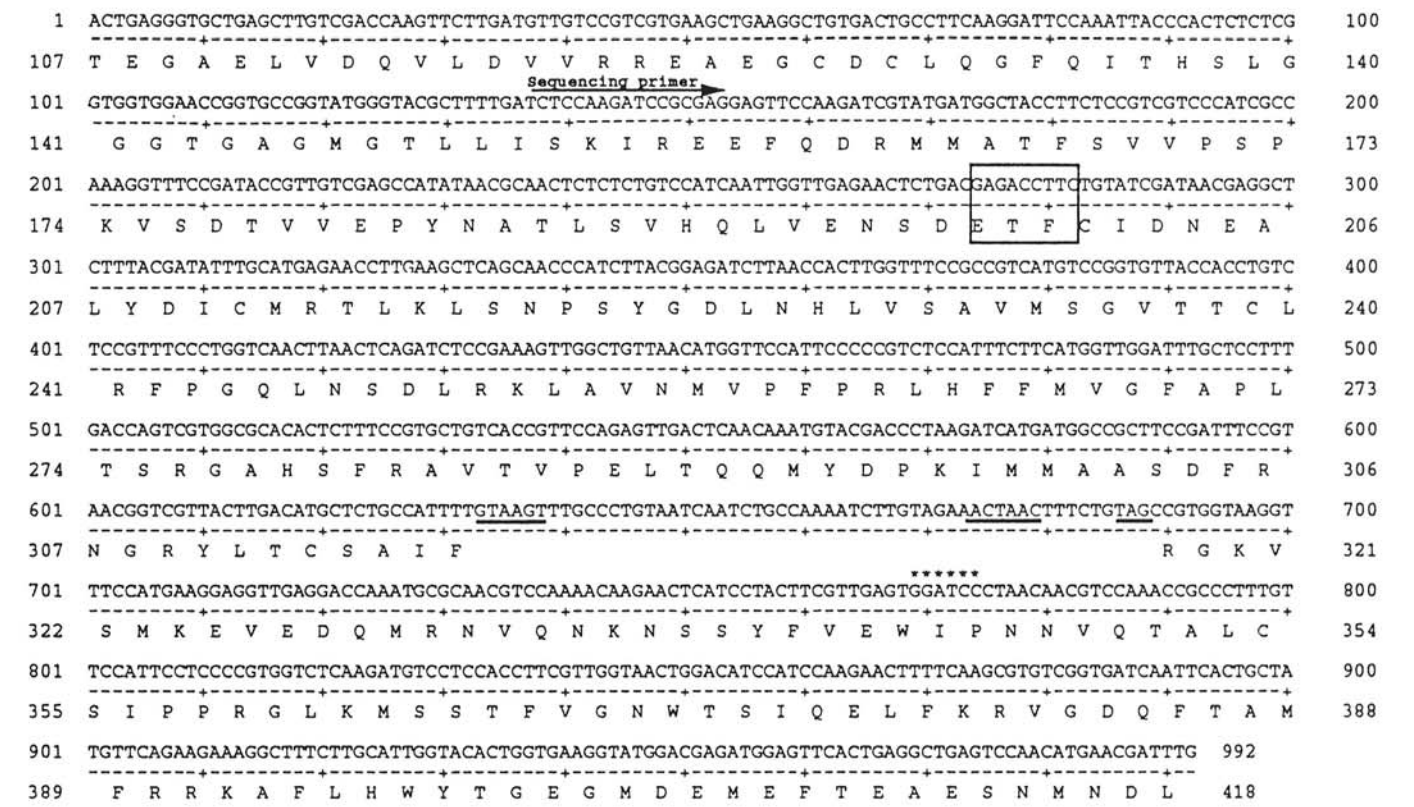

Fig. 3. Partial nucleotide sequence of the beta-tubulin gene (*benA*) from a benomyl-sensitive (wild-type) strain of *Botrytis cinerea.* The deduced amino acid sequence is indicated by the single letter amino acid code below the nucleotides. Numbers alongside nucleotide and amino acid sequences refer to the order of the nucleotides sequenced and the fungal beta-tubulin consensus amino acid alignment, respectively. The sequence corresponding to amino acids 198–200 of beta-tubulin genes from other fungi is boxed. Predicted 5′ and 3′ splice junctions and lariat formation sequences of the putative intron 6 (numbered on the basis of the beta-tubulin consensus intron designation) are underlined. The internal *Bam*HI site is marked by asterisks. The primer used for direct sequencing of the mutant alleles is marked by an arrow.

type isolate (PC9385S) and a highly benomyl-resistant strain (K1145, designated MBC$_R$) of *B. cinerea* revealed a Glu to Ala substitution at amino acid position 198 in the mutant. The nucleotide sequences of the 579-bp fragment in that study (26) and the corresponding portion of our 992-bp fragment were identical. Since the beta-tubulin gene has been only partially examined, we cannot unequivocally rule out that other segments of the gene (or elsewhere) influence the phenotype.

Comparison of the specific mutations and the corresponding phenotypes of *B. cinerea* to those found in Ben^R strains of other phytopathogenic fungi (23) are somewhat problematic, because various criteria have been used to categorize resistant phenotypes (16,23,34). Nevertheless, such comparisons show that in *B. cinerea* and in species of *Venturia* and *Penicillium,* the Ben^{MR}NPC^R phenotypes are associated with a Phe to Tyr substitution at amino acid position 200. In species of *Venturia* and *Penicillium,* amino acid substitution Glu to Lys at position 198 led to Ben^{HR}NPC^R phenotypes, whereas a Glu to Ala substitution at this position resulted in strains with very high resistance to MBC and sensitivity to NPC (23). In *B. cinerea,* both phenotypes associated with these amino acid changes at position 198 (Table 1) were defined as Ben^{HR} ($EC_{50} > 50$ μg/ml), but considering differences in their growth rates at 20 and 50 μg/ml MBC (Table 2), *B. cinerea* phenotypes Ben^{HR}NPC^R and Ben^{HR}NPC^S may well correspond to phenotypes with high resistance and very high resistance, respectively, of *Venturia* and *Penicillium.*

As in field strains of several phytopathogenic fungi, the spectrum of beta-tubulin mutations in eight Ben^R strains of *B. cinerea* was confined to amino acids 198 and 200, which further emphasizes the difference between resistant field strains and laboratory mutants (23).

Whereas three or four levels of resistance have been found in benomyl-resistant field populations of phytopathogens such as *Venturia* spp. (16,21,34), similar populations of *B. cinerea* not exposed to NPCs are composed almost exclusively of highly resistant Ben^{HR}NPC^S strains. Ben^{R}NPC^R strains have been

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B. cinerea          TEGAELVDQVLDVVRREAEGCDCLQGFQITHSLGGGTGAGMGTLLISKIREEFQDRMMATFSVVPSPKV
V. inaequalis       **************************************************P***************
N. crassa           **************************************************P***************
C. gloeosporioides  ******************T**S**************S*******A*V****P*********L*****
E. graminis         **************************************************P***************
A. nidulans (benA)  ********N*V*********************************************P***************
A. nidulans (tubC)  **********I********S*****A**V*********S**************P*********M******

B. cinerea          SDTVVEPYNATLSVHQLVENSDETFCIDNEALYDICMRTLKLSNPSYGDLNHLVSAVMSGVTTCLRFPG
V. inaequalis       ************************************************************************
N. crassa           ************************************************************VS******
C. gloeosporioides  *EV*********************************R****QAH*******K***R****L**GF****
E. graminis         ************************************************************************
A. nidulans (benA)  ******************H*****************************************************
A. nidulans (tubC)  ******************H******L**D******I******S****************I*VS******

B. cinerea          QLNSDLRKLAVNMVPFPRLHFFMVGFAPLTSRGAHSFRAVTVPELTQQMYDPKIMMAASDFRNGRYLTC
V. inaequalis       ************************************************************************S
N. crassa           ****************************************S********F*******************
C. gloeosporioides  ***A********L*********T*******T--*AAYQNLG*A******F***NV*S********F***
E. graminis         ************************************************F***N***************
A. nidulans (benA)  ********W*************************Y*****S********F***N***************
A. nidulans (tubC)  *********************************SSS***TIS********F*SRN**T*ANYQ***F***

B. cinerea          SAIFRGKVSMKEVEDQMRNVQNKNSSYFVEWIPNNVQTALCSIPPRGLKMSSTFVGNWTSIQELFKRVG
V. inaequalis       ************************************************************************
N. crassa           ********************************************************S*A*******I*
C. gloeosporioides  ***Y*****T*QI*E*I*G**A***A**************H*****V*MNA****I**S*A**DI*R***
E. graminis         ********************Q**V**********************************S***********
A. nidulans (benA)  ******************I*S**Q**********I*S****************I**S***********
A. nidulans (tubC)  *TL*****A**********M***Y******************M**K****AA*****S**V****N**S

B. cinerea          DQFTAMFRRKAFLHWYTGEGMDEMEFTEAESNMNDLV
V. inaequalis       ************************************
N. crassa           E***********************************
C. gloeosporioides  ***SV********************************
E. graminis         ************************************
A. nidulans (benA)  ************************************
A. nidulans (tubC)  N**********************************M
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Fig. 4. Comparison of deduced amino acid sequences of beta-tubulins of filamentous fungi. The deduced partial amino acid sequence of the *Botrytis cinerea* wild-type beta-tubulin protein is given on the top line. For *Venturia inaequalis* (23), *Neurospora crassa* (29), *Colletotrichum gloeosporioides* (1), *Erysiphe graminis* (36), and *Aspergillus nidulans benA* and *tubC* (27) sequences, identical amino acids are marked by asterisks, and amino acids different from those in *B. cinerea* are listed. Dashes have been inserted, when required, for optimal alignment.

extremely rare in such populations and became apparent only after the application of MBC + NPC for control of gray mold. Assuming that mutations occur randomly and at equal rates in the region encoding amino acids 198–200 of beta-tubulin, the rarity of such strains may be indicative of low fitness. Consequently, periodic removal of NPC fungicides from control programs may help to retain sensitivity to NPC in *B. cinerea* populations.

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