Mutation at β -Tubulin Codon 200 Indicated Thiabendazole Resistance in *Penicillium digitatum* Collected from California Citrus Packinghouses

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ABSTRACT

Schmidt, L. S., Ghosop, J. M., Margosan, D. A., and Smilanick, J. L. 2006. Mutation at β -tubulin codon 200 indicated thiabendazole resistance in *Penicillium digitatum* collected from California citrus packinghouses. Plant Dis. 90:765-770.

Thiabendazole (TBZ) is commonly applied to harvested citrus fruit in packinghouses to control citrus green mold, caused by *Penicillium digitatum*. Although TBZ is not used before harvest, another benzimidazole, thiophanate methyl, is commonly used in Florida and may be introduced soon in California to control postharvest decay of citrus fruit. Isolates from infected lemons and oranges were collected from many geographically diverse locations in California. Thirty-five isolates collected from commercial groves and residential trees were sensitive to TBZ, while 19 of 74 isolates collected from 10 packinghouses were resistant to TBZ. Random amplified polymorphic DNA analysis indicated that the isolates were genetically distinct and differed from each other. Nineteen TBZ-resistant isolates and a known TBZ-resistant isolate displayed a point mutation in the β -tubulin gene sequence corresponding to amino acid codon position 200. Thymine was replaced by adenine (TTC $\rightarrow$ TAC), which changed the phenylalanine (F) to tyrosine (Y). In contrast, for 49 TBZ-sensitive isolates that were sequenced, no mutations at this or any other codon positions were found. All of the isolates of *P. digitatum* resistant to TBZ collected from a geographically diverse sample of California packinghouses appeared to have the same point mutation conferring thiabendazole resistance.

Citrus green mold, caused by *Penicillium digitatum* (Pers.:Fr.) Sacc., is an important postharvest disease of fresh citrus fruit, particularly in arid growing regions (19). The fungicides most commonly used to combat this disease in California are thiabendazole (TBZ) and imazalil (IMZ). The mode of action of TBZ (2-[4-thiazolyl] benzimidazole) was shown to be the disruption of microtubule assembly in competition binding studies with carbendazim and TBZ with *P. expansum* (13). These and all benzimidazoles are commonly recognized as essential fungal anti-tubulin agents (11,12,24,29).

Benzimidazoles bind to the fungal β -tubulin molecule, as shown for *Aspergillus nidulans* (10,12,13,29), *P. expansum* (13), and *Rhynchosporium secalis* (25), disrupting microtubule assembly and interfering with cell division during mitosis. Point mutations at the site of action inhibit this conformational change that results in microtubule resistance to inhibition by TBZ (14,36). Mutations in specific amino

acids that confer benzimidazole resistance occur at codon 165 in *A. nidulans* (29), codon 198 in *P. expansum*, *P. puberulum*, *P. digitatum*, *P. viridicatum*, *Monilinia fructicola*, and *Sclerotinia homoeocarpa* (32), and 198 or 200 in *P. italicum*, *P. aurantio-griseum*, *Venturia inaequalis*, *V. pirina* (32), and *Botrytis cinerea* (44). Mutations reported to confer TBZ resistance in *P. expansum* differ and are more complex. Scholberg and coworkers (40) reported that five resistant isolates had mutations at codon 198, where the amino acid changed from glutamic acid to alanine or valine, while seven other resistant isolates had not changed at this codon and other mutations were not found. Baraldi and coworkers (1) reported mutations at codons 198 and 200 conferred resistance, although one isolate with a mutation at codon 200 was sensitive. Among resistant isolates, the mutation at codon 198 changed the amino acid from glutamic acid to alanine, valine, or lysine. One resistant isolate had a mutation at codon 167 rather than codon 198. The mechanism of benzimidazole resistance among resistant isolates of *P. digitatum* has been little studied. In a previous study, two benomyl-resistant isolates of this pathogen had mutations at codon 198 that changed the amino acid from glutamic acid to lysine (32).

TBZ has been used in California as a postharvest treatment since 1968 (19,23). Following the detection of TBZ resistance

among *P. digitatum* isolates in California (9) and elsewhere (6,7), imazalil was introduced to control resistant isolates in 1981 (20). It is currently a common practice to apply both TBZ and IMZ to citrus fruit on packinglines either in sequence or together (2-4). Prolonged use of TBZ and IMZ in packinghouses is conducive to the selection and proliferation of resistant isolates of *P. digitatum* (27). TBZ resistance was reported many times among isolates collected in packinghouses (6,7,17,21,23,26-28). While benzimidazole resistance has long been a problem in packinghouses, presumably it may already exist at a low frequency or develop in the future in California citrus groves as well. Neither TBZ nor any other benzimidazole fungicide is applied to citrus groves in California at this time. However, the benzimidazole thiophanate methyl applied in groves before harvest effectively controls postharvest decay of citrus fruit (31,38,42), and regulatory approval for its use is in progress in California. As would be expected, resistant isolates of *P. digitatum* became common among infected fruit from groves where benzimidazoles had been applied for several years to trees before harvest (33). Interestingly, some resistant isolates were found in groves where neither TBZ nor other benzimidazoles had been used (23,33).

The purpose of this study was to collect *P. digitatum* isolates from infected citrus fruits from California packinghouses and citrus groves, to assess their resistance level to TBZ, identify sensitive and resistant phenotypes, and investigate the genetic basis of TBZ resistance. Genetic fingerprinting using random amplified polymorphic DNA (RAPD analysis) ensured that those isolates selected for β -tubulin sequencing were unique genotypes. A 781-bp region of the β -tubulin gene was sequenced to identify mutations relevant to amino acid codon positions 165, 167, 198, and 200. These sequences were compared with those previously identified from fungi, including a benomyl-sensitive *P. digitatum* isolate (EMBL:D78154), and any changes in the sequence compared with their phenotypic classification.

MATERIALS AND METHODS

Collection of *P. digitatum* isolates. *P. digitatum* isolates were collected by the method of Holmes and Eckert (27) from infected lemons or oranges from six pack-

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inghouses in Southern California ($n = 50$ isolates) and four packinghouses in the Central San Joaquin Valley ($n = 24$ isolates) in 2004 and 2005. In addition, *P. digitatum* isolates were collected from locations in the Sacramento Valley, Central San Joaquin Valley, and Southern California from fallen fruit under trees in both commercial groves ($n = 24$ isolates) and residential neighborhoods ($n = 11$ isolates). A single infected fruit was collected under each sampled tree, the fruit was placed in a sterile sealable plastic bag, its location was recorded, and it was transported to the laboratory. Under aseptic conditions, conidia were collected from decay lesions using a sterile toothpick, and the conidia were transferred to potato dextrose agar (PDA; Difco Laboratories, Detroit, MI) plates, which were incubated at 24°C. A single germinated conidium was selected from this culture, transferred to fresh PDA, and cultured at 24°C.

Determination of fungicide sensitivity. An EC₅₀ value for all isolates, defined as the TBZ concentration of a fungicide that

reduced colony diameter by 50% on PDA, was determined. The pH of the PDA was 5.6 ± 0.2 in all tests. Isolates were cultured 1 week at 20°C on PDA; then conidia were dislodged from the surface of the colony with a glass rod after the addition of a small volume of sterile water with 0.05% (wt/vol) Triton X-100 surfactant, filtered through two layers of cheesecloth, transferred into a sterile flask, and the conidial suspension was adjusted with sterile water to an optical density of 0.1 at 425 nm using a spectrophotometer. The suspension contained about 10^6 conidia per ml. A 10- μ l droplet of the conidial suspension was placed on PDA (6 isolates/plate) amended with TBZ at 0, 0.05, 0.1, 1, 10, or 25 μ g ml⁻¹. After incubation for 4 days at 25°C, colony diameters were measured, and the concentration that caused a 50% reduction in colony diameter was calculated by fitting the TBZ concentrations and colony diameters to a sigmoidal curve. The experiment was repeated three times. Sensitive (S) phenotypes were those that could not grow at concentrations of 0.2 μ g ml⁻¹

TBZ after 5 days at 20°C, while resistant (R) phenotypes grew at concentrations that exceeded 10 μ g ml⁻¹. Eckert and Wild (21) stated the discriminatory concentration of TBZ to classify isolates of *P. digitatum* as resistant to TBZ was 10 μ g ml⁻¹; if an isolate was capable of growth on agar media amended with this fungicide concentration, it was poorly controlled by TBZ applied commercially at maximum rates to citrus fruit (21). Two other isolates that we did not collect were also characterized: TBZ-resistant isolate D201 (a gift of David Sorenson of Sunkist Growers) and TBZ-sensitive isolate M6R (a gift of J. W. Eckert, University of California, Riverside).

Isolation of fungal DNA. All reagents were purchased from Sigma Aldrich Co. (St. Louis, MO) unless indicated otherwise. To obtain fungal mycelia, conidia from isolates were collected from 10-day-old colonies, cultured on PDA, and then several milligrams of conidia were placed into Erlenmeyer flasks containing 15 ml of potato dextrose broth (PDB; Difco Laboratories, Detroit, MI) that contained 50 μ g ml⁻¹ each of chloramphenicol and penicillin G. After shaking for 24 h at 27°C, the mycelia were collected and placed into sterile 1.5-ml Eppendorf tubes. The tubes were submerged in liquid nitrogen for 30 s to freeze the mycelia, then thawed in a 55°C water bath and refrozen with liquid nitrogen. Immediately following freezing, the mycelia were crushed using a sterile micropestle, and 0.5 ml of warm (65°C) cetyltrimethylammonium bromide (CTAB) buffer was added that contained 1% CTAB, 700 mM NaCl, 50 mM Tris-HCl, pH 8.0, 10 mM EDTA, and 1% β -mercaptoethanol. A standard DNA extraction protocol was followed as described by Chen and Ronald (8), with slight modifications. The tubes were vortexed for 5 min after adding 0.5 ml chloroform:isoamyl alcohol (24:1), then centrifuged at $10,000 \times g$ for 10 min. The aqueous phase was added to an equal amount of room temperature isopropanol and gently mixed by inversion of the tubes several times. The tubes were centrifuged at $12,000 \times g$ for 4 min; the supernatant was poured off, and the pellet was dried in air. The pellet was washed three times in 0.5 ml of 70% ethanol and centrifuged each time at $12,000 \times g$ for 4 min. Pellets were dried for at least 15 min in air and re-suspended in 0.1 ml of Tris-EDTA (TE; 50 mM Tris-HCl, 10 mM EDTA, pH 8.0) and 20 μ g ml⁻¹ RNase. The DNA was quantified spectrophotometrically as described by Sambrook and Russell (39) and stored at -80°C.

RAPD fingerprinting. RAPD primers were obtained from Invitrogen (Carlsbad, CA). The primers G02 and I06 (18), pen (37), ar1, nor1, and omt1 (22) were selected from previous studies of *Penicillium* spp. and initially screened against genomic DNA of two *P. digitatum* isolates. Omt1

Table 1. Origin of isolates collected from California packinghouses and citrus trees and the history of thiabendazole (TBZ) use

Origin of isolates	TBZ use ^a	Total isolates	Resistant isolates
Fowler - Central San Joaquin Valley	A	10	6
Porterville - Central San Joaquin Valley	A	3	1
Terra Bella - Central San Joaquin Valley	A	3	1
Santa Paula - Southern California	A	10	8
Saticoy - Southern California	A	4	0
Ivanhoe - Central San Joaquin Valley	B	8	1
Montalvo - Southern California	B	9	0
Oxnard - Southern California	B	8	0
Saticoy - Southern California	B	9	0
Ventura - Southern California	B	10	2
Tree ^b - Sacramento Valley (Orland, Oroville area)	C	12	0
Tree ^b - Central San Joaquin Valley	C	19	0
Tree ^b - Southern California	C	4	0
Total isolates		109	19

^a A = TBZ in use at the time of collection; B = TBZ not in use at the time of collection, use in past probable; C = no history of TBZ or other fungicide use.

^b Isolates were collected from either commercial groves or residential neighborhoods.

Fig. 1. An agarose gel with individual fingerprint profiles of *Penicillium digitatum* isolates from random amplified polymorphism DNA analysis using primer omt1. Lanes 1 and 18 each contain a 100-bp marker, lane 11 is a negative control, and lanes 2 to 10 and 12 to 17 are profiles from *P. digitatum* isolates.

was selected on the basis that it generated polymorphic bands. The 25- μ l PCR mixture contained: 1 μ l (75 pmol) of primer omt1, 5' GTGGACGGACCTAGTCCG-ACATCAC 3', 0.5 μ l (5 ng) of template DNA, 2 μ l of 0.6% bovine serum albumin (BSA), 12.5 μ l of PCR Master Mix (Roche Applied Science, Indianapolis, IN), and 9 μ l of sterile distilled water. The reactions were performed in an Eppendorf Mastercycler thermocycler (Hamburg, Germany) with conditions that consisted of an initial denaturation at 96°C for 8 min followed by 42 cycles of 95°C for 1 min, 40°C for 3 min, and 72°C for 4 min, with a final extension at the end of these cycles of 72°C for 10 min. A 10- μ l volume of the product was electrophoresed on a 2% molecular biology grade agarose gel in TE buffer at 85 volts for 90 to 120 min. A 100-bp ladder (G210A; Promega, Madison, WI) was used as a standard. The gel was placed in a dilute solution of ethidium bromide (0.5 μ g ml⁻¹) for 10 min and destained in distilled water for 20 min. The bands were visualized under UV light and digitized (Alpha Imager 2000; Alpha Innotech Corp. San Leandro, CA). The RAPD analysis was repeated three times for each isolate.

Analysis of RAPD-PCR reactions. All bands were compared against a 100-bp ladder for size and scored as either 0 or 1, indicating either the absence or presence of a band, respectively. The binary data from RAPD-PCR fingerprinting grouped the isolates according to the relatedness of banding patterns using hierarchical cluster analysis with squared Euclidian distance and between groups linkage (SPSS version 11.5, SPSS Inc., Chicago, IL).

β -tubulin gene sequence. A 1,000-bp segment of the β -tubulin gene (15) was amplified, which covered the 165, 167, 198, and 200 amino acid codon positions using the forward primer Bt-T2m-Up and reverse primer Bt-LEV-Lo1 and PCR cycling parameters as described by de Jong et al. (15). Each 50- μ l reaction contained 4 μ l (40 ng) of template DNA, 1 μ l of each primer (67 pmol forward; 56 pmol reverse), 25 μ l of PCR Master Mix (Roche Applied Science, Indianapolis, IN), 4 μ l of 0.6% BSA, and 15 μ l of sterile distilled water. PCR products were electrophoresed on a 1% agarose gel, then gel purified using DNA Gel Extraction Kit (Montage, Bedford, MA), and cycle-sequenced on both strands using the PCR conditions of Jung and Oakley (29), and read on an ABI 3730 DNA Analyzer (Applied Biosystems Inc., Foster City, CA). The sequences were then compared to previously published β -tubulin sequences (GenBank, available online).

RESULTS

TBZ-S and TBZ-R phenotypes. The EC₅₀ TBZ concentrations among isolates were in two classes, less than 0.2 μ g ml⁻¹ and 10 to 25 μ g ml⁻¹, that corresponded to

Fig. 2. Hierarchical cluster analysis of random amplified polymorphism DNA banding patterns from thiabendazole (TBZ) sensitive (S) and TBZ resistant (R) *Penicillium digitatum* phenotypes collected from citrus fruit from trees or packinghouses. Isolates were scored by the presence (1) or absence (0) of bands, and the dendrogram was generated using squared Euclidean distance.

RAPD analysis. As shown by RAPD analysis using omt1, all isolates presented a common band of approximately 500 and 800 bp; however, there was a notable variation in the other bands from 175 to 500 bp in length (Fig. 1). Isolates with identical fingerprints occurred among some that were collected at the same time and location, indicating they were clones of the same isolate, and the presumed duplicate isolates were omitted from further characterization. Hierarchical cluster analysis (SPSS 11.5) of the banding patterns produced one large cluster, with many isolates presenting a unique banding pattern, which indicated a high degree of heterogeneity within the *P. digitatum* population in California (Fig. 1). Some isolates collected from distant locations from each other had similar fingerprints. For example, the fingerprints of Fowler-R, Terra Bella-S, and Orland-S were similar (Fig. 2); however, the distance between

Sequencing of the β -tubulin gene of TBZ-R and TBZ-S phenotypes. The 20 resistant phenotypes and 49 sensitive phenotypes, encompassing all collection areas and showing unique RAPD banding patterns, were chosen for sequencing of the β -tubulin gene. The selection of 49 sensitive isolates included those from packing-houses (two isolates each from Saticoy, Santa Paula, Porterville, and Terra Bella, four from Ventura, five from Oxnard, and six from Montalvo) and those from trees (three from Southern California, six from the Central San Joaquin Valley, and seven from the Sacramento Valley). A region of the β -tubulin gene, incorporating those nucleotides corresponding with amino acids 165 to 200, was sequenced on both the forward and reverse strands, using primers Bt-T2m-Up and Bt-LEV-Lo1 (15).

DISCUSSION

The importance of specific amino acids in fungal tubulin with respect to benzimi-

<i>P. digitatum</i> isolates sequenced	TBZ MIC (µg/ml) ^a	Phenotype	Nucleotide substitution	Codon position 200
49	0.05–1.00	S	None	Phe
20	10.00–20.00	R	TTC to TAC	Tyr

^a TBZ sensitive (S) and TBZ resistant (R) phenotypes had EC₅₀ TBZ concentrations of $\leq 0.2 \mu\text{g ml}^{-1}$ and $\geq 10 \mu\text{g ml}^{-1}$, respectively, on amended potato dextrose agar as determined by colony diameter.

[illegible]

^a Asterisk denotes identical residue to above sequence. A single residue change (from phenylalanine to tyrosine) is indicated at position 200 in all thiabendazole (TBZ)-R isolates, but is absent in all TBZ-S isolates.

dazole resistance has been well studied (1,29,32,34,36,40,44). All 20 TBZ-resistant isolates of *P. digitatum* identified in this study had a single mutation that changed the amino acid at codon position 200. This is the first report of TBZ resistance in *P. digitatum* conferred by a codon change at position 200 in the β -tubulin gene. This result differs from that of Koenraadt and coworkers (32), who reported that benomyl resistance in the two isolates of *P. digitatum* they examined was conferred by a mutation at codon position 198.

The tubulin gene is highly conserved, with a ~75% sequence similarity within each family (5); however, there are specific amino acid residues that are repeatedly associated with fungicide resistance (1,29,32,34,36,40,44). The relevance of the residue at codon position 200 of the tubulin gene of *Schizosaccharomyces pombe* was examined by Paluh and coworkers (36) with respect to the rate of microtubule depolymerization when the codon was changed from phenylalanine to tyrosine, the same change we observed with *P. digitatum* isolates resistant to thiabendazole. They discovered when examining the microtubule dynamics of a mutated (F200Y) *S. pombe* strain that an interruption in mitosis and a complete failure of meiosis occurred because of the delayed breakdown of the tubulin heterodimer. The crystal structure of the β -tubulin protein (35,36) displays the residue corresponding to position 200 at the internal interface where the α - and β -tubulins join to form a heterodimeric molecule, and also adjacent to the GTP binding site (44). The constant cycling of microtubules, referred to as dynamic instability (30,36), involves the polymerization and de-polymerization of the tubulin heterodimers. Many helper proteins, such as chaperones, GTPases, and kinases facilitate microtubule dynamics (41). It has also been suggested that an asymmetric distribution of these proteins, due to a delay in spindle de-polymerization during meiosis (36) caused by the mutation of F200Y, as seen in the *P. digitatum* TBZ-R isolates, could affect spindle stability. These findings suggest that the internal interface plays a crucial role in one of the following: (i) the stabilization dynamics of the α - and β -tubulin molecules; (ii) the interaction between the microtubule and GTP; or (iii) uneven distribution of the proteins used in microtubule dynamics.

Phylogenetic work by Detrich et al. (16) verified the importance of phenylalanine at residue 200, pinpointing it as the single consistent sequence difference between cold-stable microtubules (belonging to Antarctic fish) and heat-stable microtubules associated with all homeotherms, such as mammals, where the phenylalanine has been substituted with tyrosine. Fungi have cold-stable microtubules with phenylalanine at position 200; however, the mutation to tyrosine, which is common

in all heat-stable microtubules, has been associated with benzimidazole resistance (36). In addition, Wright and Hunter (43) found that cold-stability and benomyl resistance were allele specific in *Caenorhabditis elegans*. Temperature influenced the benzimidazole sensitivity of some resistant isolates of *Aspergillus nidulans* (14,32) and *Saccharomyces cerevisiae* (14). Isolates of *Monilinia fructicola*, cause of brown rot of stone fruit, that were highly resistant to benomyl lost their resistance at 31°C, while those isolates with a low level of resistance lost benomyl resistance at 15°C (34). Further studies to observe temperature effects on TBZ-R isolates of *P. digitatum* could influence strategies to manage resistant isolates in packinghouses.

Some conclusions from our work include: (i) RAPD patterns of TBZ-R isolates indicated *P. digitatum* populations may be heterogeneous, although more work is needed to analyze diversity in populations of this pathogen. If heterogeneous before TBZ resistance developed, resistance may have developed many times independently and is not the result of a rare mutation that dispersed throughout California. Conversely, TBZ resistance may have developed only once and the diversity among the isolates we observed occurred later; (ii) it is likely a single point mutation at codon 200 of the β -tubulin gene is responsible for the reduction in competitive fitness associated with benzimidazole resistance that was reported previously (21,26), where resistant isolates of *P. digitatum* collected in California declined rapidly in populations of conidia that developed from citrus fruit co-inoculated with a mixture of benzimidazole-resistant and -sensitive conidia; and (iii) the development of a molecular probe to detect the single point mutation at codon 200 of the β -tubulin gene of *P. digitatum* is feasible and would facilitate the detection of resistant isolates. The detection of resistant isolates will become particularly important now to monitor TBZ resistance in packinghouses and in the future in groves if grove applications of thiophanate methyl become popular in California. Presumably, isolates resistant to TBZ will also be resistant to thiophanate methyl, but this should be confirmed, because isolates of other *Penicillium* spp. have been resistant to one but not both fungicides (40). Rigorous monitoring to detect benzimidazole resistance is needed to identify isolates resistant both to this fungicide and to TBZ in coordinated strategies to minimize their development.

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