Genotyping of Benzimidazole-Resistant and Dicarboximide-Resistant Mutations in *Botrytis cinerea* Using Real-Time Polymerase Chain Reaction Assays

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ABSTRACT

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Botrytis cinerea, an economically important gray mold pathogen, frequently exhibits multiple fungicide resistance. A fluorescence resonance energy transfer-based real-time polymerase chain reaction assay has been developed to detect benzimidazole- and dicarboximide-resistant mutations. Three benzimidazole-resistant mutations—¹⁹⁸Glu to Ala (E198A), F200Y, and E198K—in β -tubulin *BenA* were detected using a single set of fluorescence-labeled sensor and anchor probes by melting curve analysis. Similarly, three dicarboximide-resistant mutations—I365S, V368F plus Q369H, and Q369P—in the histidine kinase *BcOSI* were successfully distinguished. Unassigned melting profiles in *BenA* genotyping

assay resulted in the identification of a new benzimidazole-resistant *BenA* E198V mutation. This mutation conferred resistance to carbendazim as do E198A and E198K mutations. The isolates with *BenA* E198V mutation showed a negative cross-resistance to diethofencarb, but to a lesser extent than the E198A mutants. A survey of 210 *B. cinerea* field isolates revealed that most of benzimidazole-resistant isolates possessed the E198V or E198A mutation in the *BenA* gene, and the I365S mutation in the *BcOSI* gene was also frequently observed in Japanese isolates. However, benzimidazole-resistant isolates with *BenA* F200Y or E198K mutations, which confer the diethofencarb-insensitive phenotype, were rare. Our *BenA* and *BcOSI* genotyping is a rapid and reliable method that is suitable for monitoring the fungicide-resistant field population.

Additional keywords: *Botryotinia fuckeliana*.

Botrytis cinerea (anamorph of *Botryotinia fuckeliana* (de Bary) Whetzel) causes gray mold on many economically important vegetable, fruit, and ornamental plants. The chemical control of gray mold in different crops has been adversely affected by the development of fungicide resistance in *B. cinerea*. Isolates of *B. cinerea* that are resistant to benzimidazole (e.g., carbendazim, benomyl, and thiophanate-methyl) and dicarboximide (e.g., iprodione and procymidone) fungicides are distributed worldwide (3,15,28). Benzimidazoles inhibit the assembly of microtubules by binding to β -tubulin in fungi (6), and in most cases, mutations at amino acid positions 198 and 200 in the β -tubulin gene confer benzimidazole resistance in field isolates of many plant pathogens (1,14,18,27). The *N*-phenylcarbamate diethofencarb is inactive against benzimidazole-sensitive strains but exhibits fungicidal activity against resistant strains with glutamic acid at position 198 to glycine (E198G) or E198A mutations in the β -tubulin gene by binding to the mutant β -tubulin (7,8,11). Three types of benzimidazole-resistant strains of *B. cinerea* have been isolated from fields; mutations within the β -tubulin *BenA* gene of these isolates produced different phenotypes (27). The *BenA* E198A mutant strains were detected worldwide as a major benzimidazole-resistant strain in fields; they are highly resistant to the benzimidazole

carbendazim and sensitive to the negative cross-resistant fungicide diethofencarb. After the introduction of a mixture of diethofencarb and carbendazim (or thiophanate-methyl) to control gray mold, strains with the F200Y or E198K mutation that are resistant to both benzimidazole and diethofencarb have been isolated from fields (15,27). Strains with the F200Y mutation are moderately resistant to benzimidazoles, while the E198K mutants like the E198A mutants are highly resistant to benzimidazoles (27). On the other hand, dicarboximides exert their antifungal toxicity by interfering with a histidine-kinase signal transduction pathway (2,9,10,21–23). Mutations in the osmosensor-like histidine kinase *BcOSI* gene result in a dicarboximide-resistant phenotype in *B. cinerea*, and strains possessing the I365S mutation in *BcOSI* are dominant (5,15,19,25,26). Recently, we scored the sensitivity to iprodione, a dicarboximide fungicide, in *B. cinerea* field isolates collected from several distantly located fields in Japan and identified two additional types of resistant isolates in Osaka area along with the I365S mutant isolates (designated as type I). A group of isolates designated type II carried mutations that yield three amino acid substitutions (V368F, Q369H, plus T447S), and the other group designated type III carried mutations causing two amino acid substitutions (Q369P plus N373S) in the *BcOSI* gene (25). These three types of dicarboximide-resistant isolates possessed similar levels of resistance and pathogenicity.

A rapid and convenient method to detect fungicide resistance is vital for fungicide resistant management. Advances in molecular biology have provided new opportunities for rapidly detecting

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fungicide-resistant genotypes once the mechanisms of resistance have been elucidated at a molecular level. Several molecular techniques such as allele-specific (AS)-polymerase chain reaction (PCR), PCR-restriction fragment length polymorphism (RFLP), and primer-introduced restriction enzyme analysis (PIRA)-PCR have been reported (13,17,25). These PCR-based methods are more convenient than the traditional antifungal susceptibility test, if only one mutation confers the fungicide resistance. However, at least three types of mutations in the *BenA* and *BcOS1* genes confer resistance to benzimidazoles and dicarboximides, respectively, in *B. cinerea*. In this study, we present single-tube assay protocols for detecting mutations that cause resistance to multiple benzimidazoles as well as dicarboximides. Two adjacent oligonucleotide probes whose fluorescent labels communicate through fluorescence resonance energy transfer are used to recognize a change or changes in the nucleotide sequence of the template DNA by analyzing melting curve profiles on a real-time PCR instrument. The objective of this study was to use this method and (i) analyze the distribution of mutations that confer *B. cinerea* resistance to carbendazim or iprodione in Japan, and (ii) isolate a novel type of benzimidazole-resistant strain that is widely distributed and dominant in Japanese fields.

MATERIALS AND METHODS

Strains and culture condition. A total of 210 field isolates of *B. cinerea* were used in this study: 105 isolates were previously collected from 26 fields in Japan during 2000 as described previously (25) and the other 105 isolates were newly collected from seven fields in Osaka Prefecture in 2002 (19 isolates), 2003 (21 isolates), 2004 (41 isolates), and 2005 (24 isolates). The five *B. cinerea* strains listed in Table 1 were selected to design the hybridization probe-based PCR protocols for *BenA* and *BcOS1* genotyping. The strain Bc-05-k11 was isolated in Kanagawa Prefecture in 2005. These strains were isolated either from eggplant, tomato, or cucumber plants. *B. cinerea* strains were maintained on potato dextrose agar (PDA) after single colony isolation.

DNA extraction and sequencing. To extract genomic DNA from the *B. cinerea* isolates, mycelial disks were cultured on cellophane sheets placed on PDA medium at 18°C for 3 days.

Mycelia were collected and homogenized with a bead beater (FastPrep Instruction, Qbiogene, Carlsbad, CA) for 30 s in a lysis buffer (10 mM Tris-HCl [pH 8.0], 0.1 M EDTA, and 0.5% [wt/vol] sodium dodecyl sulfate) and 0.4 g of glass beads (1.0 mm diameter). The resultant sample was incubated at 65°C for 30 min. After phenol extraction and isopropanol precipitation, the genomic DNA thus obtained was used as a template for PCR or real-time PCR. DNA sequence analysis was conducted using an ABI Model PRISM310 Auto Sequence System (Applied Biosystems, Foster, CA) and fluorescent dye terminator dideoxynucleotides according to the PCR cycle sequencing protocol provided by the manufacturer. The primer sets for DNA sequencing and PCR amplification were TUB-F1 (5'-GCTTTTGATCTCCA-AGATCCG-3'; nucleotide positions, 1757 to 1777) and TUB-R1 (5'-CTGGTCAAAGGAGCAAATCC-3'; nucleotide positions, 2134 to 2115) for *BenA* (accession no. Z69263) and BcOS1-F1 (5'-TAGCTCATGGAGATCTCACACA-3'; nucleotide positions, 2010 to 2031) and BcOS1-R1 (5'-GACTTCTCGCGCAAATT-GTTG-3'; nucleotide positions, 2401 to 2381) for *BcOS1* (accession no. AF435964).

Real-time PCR using fluorescent hybridization probes. Genotyping of benzimidazole- and dicarboximide-resistant mutations was performed by a LightCycler (LC) instrument (Roche Diagnostics, Basel, Switzerland) with specific sets of primers and hybridization probes (Table 2). These probes consist of two different oligonucleotides—one labeled at the 5' end with the acceptor fluorophore LC-Red 640 and one labeled at the 3' end with the donor fluorophore fluorescein. When these probes hybridize to two adjacent internal sequences of the PCR-amplified products and the light source of the LC excites fluorescein, a fluorescent resonance energy transfer occurs between fluorescein and LC-Red 640. This results in the emission of fluorescence, which is measured by the instrument. Mutations can be determined by melting curve analyses. Real-time PCR with the hybridization probe was performed in glass capillaries in a total volume of 20 µl in the LC system (Roche Diagnostics). Each reaction mixture contained an appropriate amount of genomic DNA, 1× LC DNA Master Hybridization Probes mix (Roche Diagnostics), 3 mM MgCl₂, 0.5 µM of each primer, 0.2 µM of the probe labeled with fluorescein, and 0.25 µM of the probe labeled with LC-Red 640. The PCR amplification comprised an initial denaturation step

TABLE 1. List of representative isolates of *Botrytis cinerea* used in this study

Isolate	Sensitivity to carbendazim ^a	Sensitivity to diethofencarb ^b	Amino acid substitution within β-tubulin	Sensitivity to iprodione ^c	Amino acid substitutions within BcOS1
Bc-56	S	R	...	S	...
Bc-45	R	S	E198A	R	I365S
Bc-05-k11	MR	R	F200Y	S	...
Bc-M9-3	R	R	E198K	R	V368F, Q369H, T447S
Bc-o-9	R	S	E198A	R	Q369P, N373S

^a S, sensitive to carbendazim at 0.1 µg/ml; MR, resistant to carbendazim at 0.1 µg/ml but sensitive to carbendazim at 25 µg/ml; and R, resistant to carbendazim at 25 µg/ml.

^b S, sensitive to diethofencarb at 25 µg/ml; and R, resistant to diethofencarb at 25 µg/ml.

^c S, sensitive to iprodione at 0.4 µg/ml; and R, resistant to iprodione at 0.4 µg/ml.

TABLE 2. Sequence of the primers and hybridization probes used in the real-time polymerase chain reaction assay of *Botrytis cinerea* isolates

Probe and primer	Sequence (5'→3') ^a	Nucleotide position ^b	Target gene
TUB-HPF1	TGTCGAGCCATATAACGCAA	1847–1866	<i>BenA</i>
TUB-HPR1	CCAACCTTCGGAGATCTGAG	2051–2070	<i>BenA</i>
TUB-U27198-Sn	TGAGAACTCTGACGCGACCTTCTGTAT-FL	1889–1915	<i>BenA</i>
TUB-U27198-An	RED-GATAACGAGGCTCTTTACGATATTGCGATGAGAACC	1917–1952	<i>BenA</i>
BcOS1-HPF1	AGCTCATGGAGATCTCACAC	2011–2030	<i>BcOS1</i>
BcOS1-HPR1	ATTGGCCATAGCGTTGACGTT	2219–2239	<i>BcOS1</i>
BcOS1-An1	GTAGGAACTGAAGGTATTCTTGGGGGTCAAG-FL	2144–2174	<i>BcOS1</i>
BcOS1-Sn1	RED-AGAAATCGAAGGCGTCCAGG	2176–2195	<i>BcOS1</i>

^a FL, fluorescein labeled; RED, LC Red 640 labeled.

^b Accession number of the *BenA* (β-tubulin) and the *BcOS1* (histidine kinase) gene were Z69263 and AF435964, respectively.

(95°C for 30 s) followed by 50 cycles of amplification (95°C for 10 s, 47°C for 10 s, and 72°C for 5 s) and a melting curve step (denaturation at 95°C for 2.5 min; annealing at 40°C for 1 min; and melting, increasing temperature to 95°C with a slope of 0.1 [°C/s]).

Assays for fungicide sensitivity and pathogenicity. To determine the sensitivity to fungicides, mycelial disks (5 mm diameter) precultured on PDA medium were placed on PDA and then amended with carbendazim, diethofencarb, or iprodione and cultured at 18°C for 3 days. The mean colony diameter minus the diameter of the inoculation disk was then measured and expressed as a percentage of the mean colony diameter of the untreated control. To examine the pathogenicity of *B. cinerea* isolates, mycelial disks (5 mm diameter) were placed on detached second leaves of cucumber (cv. Sagamihanjirō) as described previously (25) and incubated at 18°C for 3 days in a humid, dark chamber. The spreading lesions of *B. cinerea* infection were then photographed.

RESULTS

Hybridization probe assay to detect three types of benzimidazole-resistant *BenA* mutations. It is known that three types of amino acid changes in β -tubulin—¹⁹⁸Glu to Ala (E198A) or Lys (E198K), and ²⁰⁰Phe to Tyr (F200Y)—confer benzimidazole resistance in field isolates of *B. cinerea* (27). We screened our stock collection and selected three resistant strains (i.e., Bc-45, Bc-M9-3, and Bc-05-k11) possessing the mutations E198A, E198K, and F200Y, respectively, along with a sensitive strain (i.e., Bc-56) without any relevant mutations in the *BenA* or *BcOS1* genes. Their sensitivities to carbendazim, a benzimidazole, and diethofencarb, an *N*-phenylcarbamate, were associated with the mutation in β -tubulin (Table 1) and were essentially identical to those described by Yarden and Katan (27). The strains Bc-45 (E198A) and Bc-M9-3 (E198K) grew on the medium containing carbendazim at 25 μ g/ml, while the strain Bc-56 was sensitive to carbendazim at 0.1 μ g/ml. In contrast, the strain Bc-05-k11 (F200Y) was moderately resistant to carbendazim because it grew on the medium containing carbendazim at 0.1 μ g/ml but not carbendazim at 25 μ g/ml (Table 1). The strain Bc-45 was sensitive to diethofencarb at 0.1 μ g/ml, but the other strains including the sensitive Bc-56 strain were highly resistant to diethofencarb.

We indicated that amino acid substitutions associated with the benzimidazole-resistant phenotype occur at amino acid positions 198 and 200 in β -tubulin, and their mutation sites are not more than 9 bases in length. The presence of mutations within a narrow region implied that a single hybridization probe can cover these mutation sites and distinguish each mutation by analyzing the melting curve profiles after PCR amplification of the target sequence followed by hybridization. We designed PCR primers and hybridization probes to detect the *BenA* mutations in real-time PCR as shown in Table 2 and Figure 1A. The primers TUB-HPF1 and TUB-HPR1 were designed to specifically amplify a 224-bp region of the *BenA* gene that includes the 198 and 200th codons. A sensor probe (i.e., TUB-U27198-Sn) labeled at the 3' end with fluorescein can be hybridized to a region containing the mutation sites, and an anchor probe (i.e., TUB-U27198-An) labeled at the 5' end with LC-Red 640 can be hybridized 1 bp from the sensor probe. Therefore, two fluorophores localize close enough to yield a fluorescent resonance energy transfer. When a thermal analysis was performed using each type of the *BenA* gene, the melting peak temperature depended on the homology between the sensor probe and the amplified PCR product. As shown in Figure 1B, this sensor probe produced discernible melting peaks at 68.7, 62.0, 59.4, and 57.2°C for the *BenA* genes bearing the E198A mutation (GAG→GCG), the wild-type sequence, the E198K mutation (GAG→AAG), and the F200Y mutation (TTC→TAC), respectively. The PCR fragments containing the E198A mutation

(a perfect match with the sensor probe) formed the most stable hybrid with the sensor probe and showed the highest melting peak temperature. Utilizing another sensor probe with a wild-type sequence failed to produce discernible melting curve profiles (data not shown). These data indicate that the hybridization probe assay using real-time PCR is a simple and rapid method to detect multiple benzimidazole-resistant mutations in a single PCR reaction if the hybridization probes are properly designed.

Hybridization probe assay to detect three types of dicarboximide-resistant *BcOS1* mutations. Mutations in the *BcOS1* gene that encode a histidine kinase confer dicarboximide resistance to field isolates (5,15,19,26). We previously reported that the strains with the ³⁶⁵Ile to Ser (I365S) mutation (type I) were dominant in Japan; however, two other types of resistant strains, represented by Bc-M9-3 and Bc-o-9, were isolated mainly from the Osaka area (25). These two strains have three amino acid substitutions [³⁶⁸Val to Phe (V368F), ³⁶⁹Gln to His (Q369H), plus ⁴⁴⁷Thr to Ser (T447S) (type II)] and two amino acid substitutions [³⁶⁹Gln to Pro (Q369P) plus ³⁷³Asn to Ser (N373S) (type III)], respectively, in BcOS1p (Table 1). Most of known dicarboximide-resistant field strains have mutation(s) in the region between the 365th and the 369th codon of the *BcOS1* gene, suggesting that mutations in this region confer dicarboximide resistance in *B. cinerea*. Therefore, we designed a sensor probe, i.e., BcOS1-Sn1, labeled at the 5' end with LC-Red 640 that surveys the region containing three types of mutations—I365S (ATC→AGC), V368F (GTC→TTC) plus Q369H (CAG→CAC), and Q369P (CAG→CCG) (Fig. 1C). After PCR amplification of the target sequence of the *BcOS1* gene with the primers BcOS1-HPF1 and BcOS1-HPR1, thermal analyses were performed using the hybridization probes BcOS1-Sn1 and BcOS1-An1. Melting curve peaks were detected at 65.7°C (Bc-56, wild-type), 62.5°C (Bc-o-9, Q369P), 59.2°C (Bc-45, I365S), and 54.5°C (Bc-M9-3, V368F, and Q369H) (Fig. 1D). BcOS1-Sn1, whose sequence was identical to that of the wild-type *BcOS1* gene, formed the most stable hybrid with the PCR products amplified from the wild-type *BcOS1* gene and formed the most unstable hybrid with the PCR products amplified from the *BcOS1* gene in Bc-M9-3. These data indicate that this hybridization probe assay using real-time PCR is useful to detect not only the mutations responsible for benzimidazole resistance but also those responsible for dicarboximide resistance.

Genotyping of *B. cinerea* field isolates revealed a wide distribution of a novel type of benzimidazole-resistant strain in Japan. A total of 105 *B. cinerea* field isolates were collected from 26 fields in nine distantly located regions in Japan (25). We attempted to classify the *BcOS1* and *BenA* genotypes of these 105 isolates using newly designed hybridization probe assay protocols. In *BcOS1* genotyping, the melting peak temperature, obtained from the genomic DNA of each isolate used as a template, matched that from one of four standard *BcOS1* genes (sensitive and three types of mutants) without exception, and the mutations in the *BcOS1* genes were identical to those determined by DNA sequencing, PCR-RFLP, and/or AS-PCR methods, as previously described (25). However, the presence of an unassigned melting peak at 63.4°C in the *BenA* genotyping assay (Fig. 1B) suggested the presence of another type of mutation in the *BenA* gene that may confer resistance to benzimidazole. DNA sequencing analysis revealed that the *BenA* genes with the unassigned peak under the melting curve assay had a point mutation (GAG→GTG) at codon 198 in which glutamic acid was substituted by valine (E198V). Six isolates with the *BenA* E198V mutation were selected (Bc-c13-3 and Bc-c13-7 from Chiba, Bc-o-36 and Bc-o-18 from Osaka, Bc-k-18 from Kochi, and Bc-m-1 from Miyazaki) and the sensitivity to carbendazim, diethofencarb, and iprodione (Table 3) was examined. All isolates with the *BenA* E198V mutation grew well on a medium amended with carbendazim at 25 μ g/ml, similar to the strains with the E198A or E198K muta-

tion, suggesting that glutamic acid at codon 198 is essential for sensitivity to benzimidazoles. Negative cross-resistance to diethofencarb was observed in the isolates with the *BenA* E198V mutation; however, they were less sensitive to diethofencarb compared with the strains with the *BenA* E198A mutation (Table 3). The values of EC₅₀ (concentration required to inhibit radial growth by 50%) for diethofencarb ranged from 0.7 to 0.8 µg/ml in the six examined isolates with *BenA* E198V while that for the Bc-45 strain with *BenA* E198A was 0.1 µg/ml. The Bc-o-18 isolate with the *BenA* E198V mutation infected cucumber leaves, and its lesions expanded in high relative humidity (>95% RH) (Fig. 2). The size of the lesions in the Bc-o-18 isolate was very similar to

those of the benzimidazole-sensitive and -resistant strains, suggesting that strains with the *BenA* E198V mutation have a pathogenicity similar to that of other strains. The E198V mutation in the *BenA* gene had no apparent effect on the sensitivity to iprodione. Two isolates (Bc-c13-3 and Bc-o-36) possessing the wild-type *BcOS1* gene and four isolates (Bc-c13-7, Bc-o-18, Bc-k-18, and Bc-m-1) with the I365S mutation showed sensitivities similar to those of the Bc-56 (sensitive to iprodione) and Bc-45 (resistant to iprodione) strains, respectively (Table 3).

A devised genotyping assay protocol was used to survey the occurrence of benzimidazole-resistant *BenA* mutations in *B. cinerea* in Japan (Table 4). Among the 105 field isolates, 70 isolates were

Fig. 1. Real-time polymerase chain reaction using fluorescent hybridization probes and melting curve analysis. **A**, DNA sequence of the sensor probe and mutations conferring benzimidazole resistance in the β -tubulin *BenA* gene. Hyphens (-) indicate nucleotides identical to those in the sensor probe. The sensor probe TUB-U27198-Sn is a perfect match to the *BenA* E198A gene. Therefore, the wild-type *BenA* gene and the F200Y, E198K, and E198V mutants have a single mismatch, i.e., C→A, T→A, G→A, and C→T, respectively. **B**, Derivative melting curves [-(dF/dT) versus T] of the wild-type *BenA* gene and the four types of benzimidazole-resistant mutations for the *BenA* genes. The fluorescence signal (F) was continuously monitored during the temperature ramp and then plotted against temperature (T) to obtain the melting curves for the samples (F versus T). The melting curves were subsequently converted to derivative melting curves [-(dF/dT) versus T]. The melting peaks of the wild-type *BenA* gene and those for the E198A, E198V, E198K, and F200Y mutants were at 62.0, 68.7, 63.4, 59.4, and 57.2°C, respectively. **C**, DNA sequence of the sensor probe and the mutations conferring dicarboximide resistance in the *BcOS1* gene. Hyphens (-) indicate nucleotides identical to those of the sensor probe. The sensor probe BcOS1-Sn1 is a perfect match to the wild-type (sensitive) *BcOS1* gene. The *BcOS1* genes with the I365S and Q369P mutations have single mismatches, i.e., T→G and A→C, respectively. The *BcOS1* gene with the V368F plus Q369H mutations has two mismatches, i.e., G→T and G→C. **D**, Derivative melting curve of the wild-type *BcOS1* gene and three types of dicarboximide-resistant mutations in the *BcOS1* gene. The melting peaks of the wild-type *BcOS1* gene and the Q369P, I365S, and V368F plus Q369H mutations were at 65.7, 62.5, 59.2, and 54.5°C, respectively.

TABLE 3. Sensitivity to fungicides in benzimidazole-resistant isolates of *Botrytis cinerea* with E198V in *BenA* gene

Isolate	Amino acid substitution	Sensitivity to fungicides ^a		
		Carbendazim	Diethofencarb	Iprodione
Bc-56	Sensitive	0.1	>25	0.2
Bc-45	E198A	>25	0.1	2.6
Bc-05-k11	F200Y	6.6	>25	0.2
Bc-M9-3	E198K	>25	>25	2.2
Bc-c13-3, Bc-o-36	E198V	>25	0.7	0.2
Bc-c13-7, Bc-o-18, Bc-k-18, Bc-m-1	E198V	>25	0.7–0.8	2.0–2.5

^a Concentration that inhibits radial growth by 50% (EC₅₀).

resistant to carbendazim, 32 isolates possessed the E198A mutation, 37 isolates possessed the E198V mutation, and one isolate possessed the E198K mutation. This result indicated that E198V and E198A were two major benzimidazole-resistant mutations. The strains carrying these two mutations were widely distributed in Japan, whereas strains with the E198V mutation were not found in Hokkaido Prefecture, a northern island of Japan (data not shown). On the other hand, we found only one isolate with the E198K mutation and no isolate with the F200Y mutation in 105 isolates. We have reported that these 105 *B. cinerea* field isolates can be grouped into four categories according to their *BcOS1* genotype by procedures based on PCR-RFLP and AS-PCR (25). The combination of *BenA* and *BcOS1* genotyping assays classified *B. cinerea* into 20 groups. Twenty-seven isolates with the *BenA* E198V mutation had the *BcOS1* I365S mutation, and 10 other isolates with the E198V mutation had no mutation in the *BcOS1* gene and were sensitive to dicarboximide. No *BcOS1* Q369P or V368F plus Q369H mutants were found in the *BenA* E198V mutants.

Regional survey of genotype changes in *B. cinerea*. We previously reported that dicarboximide-resistant strains with the *BcOS1* I365S mutation were distributed throughout Japan, but other types of resistant strains with the *BcOS1* Q369P and *BcOS1* V368F plus Q369H mutations were almost specifically found in fields in Osaka during 2000 (Table 4) (25). To determine the stability of these unique dicarboximide-resistant strains in the Osaka

area, we further obtained a total of 105 *B. cinerea* strains (40 isolates between 2002 and 2003 and 65 isolates between 2004 and 2005). Mutations within the *BcOS1* and *BenA* genes were determined using hybridization probe assay procedures described previously (Table 5). The proportion of dicarboximide-sensitive isolates was 17% in 2000, 43% from 2002 to 2003, and 45% from 2004 to 2005. The increasing frequency of dicarboximide-sensitive isolates probably come from the reducing usage of dicarboximide fungicides by introduction of new fungicides such as phenylpyrroles, anilinopyrimidines, and hydroxyanilides. An increased proportion of dicarboximide-sensitive isolates in the Osaka area mainly resulted from an increase in the number of isolates that were sensitive to both carbendazim and iprodione. The strain bearing the *BcOS1* I365S mutation was a major dicarboximide-resistant population in every period. The *BcOS1* I365S mutation was only found in benzimidazole-resistant isolates, and most of these isolates also carried the *BenA* E198V mutation. The proportion of the *BcOS1* V368F plus Q369H isolates in the total isolates decreased 34% (10 of 29) in 2000, 13% (5 of 40) from 2002 to 2003, and 6% (4 of 65) from 2004 to 2005. Most of the *BcOS1* V368F plus Q369H isolates (16 of 19) were sensitive to carbendazim. Meanwhile, the ratio of the number of benzimidazole-sensitive to the total number of isolates was stable at 32 to 35% from 2000 to 2005. Only two isolates possessing the *BcOS1* Q369P mutation along with the E198A mutation in the *BenA* gene had been isolated in 2000 and were not isolated thereafter. The strain with the *BcOS1* Q369P and the *BenA* E198V mutations was not isolated in 2000 but became dominant as its proportion increased to 31 and 28% in the benzimidazole-resistant isolates between 2002 and 2003 and 2004 and 2005, respectively. Moreover, all isolates with the *BcOS1* Q369P mutation carried the *BenA* E198V mutation. These results indicated that the dicarboximide-resistant strains with the *BcOS1* V368F plus Q369H and the *BcOS1* Q369P mutations appeared in different strains with the *BenA* genotype in the fields in Osaka. The benzimidazole-resistant strains with the E198K or F200Y mutation in the *BenA* gene were also seldom isolated, suggesting that these types of benzimidazole-resistant strains exist at a very low rate.

DISCUSSION

We have developed hybridization probe assay procedures for the rapid identification of base substitutions in the β -tubulin *BenA* gene and the histidine kinase *BcOS1* gene that are associated with resistance to benzimidazole and dicarboximide fungicides in *B. cinerea*, respectively. Our method is based on the hybridization property of DNA and a fluorescent resonance energy transfer between two fluorophores. Fluorescence emission occurs if two hybridization probes (sensor and anchor) are closely located. Anchor probes are designed to hybridize to the template DNA at a higher melting temperature than sensor probes. In thermal analysis, a hybrid between the sensor probe and the template with base substitution(s) results in an unstable hybrid that becomes dehybridized at a lower temperature. The peak of the melting curve that is drawn differentiating fluorescence and time versus temperature depends on the homology of the probe and the template. The position of a base substitution may influence the stability of

Fig. 2. Pathogenicity of *Botrytis cinerea* with benzimidazole-resistant mutant alleles on detached cucumber leaves. The sensitive (Bc-56) and benzimidazole-resistant Bc-45 (E198A), Bc-M9-3 (E198K), and Bc-o-18 (E198V) isolates were incubated on potato dextrose agar plates at 18°C for 7 days. Their mycelial disks (5 mm diameter) were placed on the detached first leaves of cucumber and incubated at 18°C for 3 days in a humid, dark chamber.

TABLE 4. Genotyping of benzimidazole- and dicarboximide-resistant isolates of *Botrytis cinerea* in Japan

<i>BcOS1</i> genotype	Number of isolates with <i>BenA</i> mutation ^a					Total
	Wild type	E198A	F200Y	E198K	E198V	
Wild type	24 (2)	15 (2)	0	0	10 (1)	49 (5)
I365S	3	13 (2)	0	0	27 (10)	43 (12)
V368F, Q369H	8 (8)	2 (2)	0	1	0	11 (10)
Q369P	0	2 (2)	0	0	0	2 (2)
Total	35 (10)	32 (8)	0	1	37 (11)	105 (29)

^a Numbers of Osaka isolates are indicated in parenthesis.

the hybrid, and even a base change at the same position can alter the stability. In fact, the *BenA* E198A and E198V mutations each possess a base change at the same position, and the hybridization of the TUB-U27198-Sn sensor probe with the E198A sequence distinguishes between the wild-type *BenA*, the *BenA* E198V, and the *BenA* E198A genes (Fig. 1A and B).

Recently, several PCR-based procedures for the detection of fungicide-resistant mutations have been successfully used to identify mutations conferring resistance to fungicides, including benzimidazoles and dicarboximides, in many plant pathogens (12, 18,19,25). We also previously reported two PCR techniques for the detection of the dicarboximide-resistant *BcOS1* mutation in *B. cinerea*; the I365S mutation was detected by PCR-RFLP and the V368F plus Q369H mutations could be detected by AS-PCR. In addition, the Q369P plus N373S mutations were identified by direct DNA sequencing (25). As described herein, three types of dicarboximide-resistant isolates with mutation(s) in the *BcOS1* gene and four types of benzimidazole-resistant isolates with mutation(s) in the *BenA* genes were found in Japanese fields. As we need to detect each type of mutation with less effort, preferably in a single reaction, the hybridization probe assays developed in this study for *BcOS1* and *BenA* genotyping have clear advantages over the PCR-RFLP and/or AS-PCR procedures. In a PCR-based hybridization probe methodology, the amplification of template DNA and the detection of mutations are carried out in a single tube with no additional manipulations such as restriction digestion and gel electrophoresis after the PCR set-up. Furthermore, this method can identify the presence of unidentified mutation. In addition, genotyping based on the melting curve analysis could clearly detect the individual mutations from a mixture of DNAs and estimate the ratios of strains, if the difference of peak temperature is more than 5°C. For example, a mixture of DNA from dicarboximide-sensitive (wild-type) and -resistant (I365S) isolates would display two melting peaks and the intensities depend on the population of their source. Their melting temperatures are in agreement with those from the individual *BcOS1* genotype assays (S. Banno and M. Fujimura, unpublished data).

A novel benzimidazole-resistant mutation, E198V, in the *BenA* gene was identified in *B. cinerea*. This mutation was initially discovered at an unassigned melting curve peak during *BenA* genotyping (Table 3). Changes at codons 198 and 200 in the β -tubulin gene are responsible for benzimidazole resistance in most field isolates of plant-pathogenic fungi (14,15,19,20). The E198A and E198K mutations were found in the benzimidazole-resistant isolates of many plant pathogens; however, the E198V mutation was reported only in benzimidazole-resistant *Penicillium expansum* (14).

Mutational analysis of the β -tubulin *Bml* gene in *Neurospora crassa* indicated that a glutamic acid residue at position 198 of β -tubulin has the size and charge required for binding to the benzimidazoles, and a smaller amino acid and loss of polarity facilitates diethofencarb binding to β -tubulin (8). *B. cinerea* strains with the E198V mutation as well as the E198A and E198K mutations were highly resistant to the benzimidazole carbendazim (Table 3). Interestingly, *B. cinerea* isolates with the E198V mutation were distributed widely in Japan (Table 5). It is known that the application of benzimidazoles to control gray mold leads to the appearance of resistant isolates with the E198A mutation that show negative cross-resistance to diethofencarb. The application of a mixture of carbendazim (or thiophanate-methyl) and diethofencarb may yield strains with the E198K or F200Y mutations in the *BenA* gene (15). *B. cinerea* isolates with the E198K or F200Y mutations were barely found in our survey, even though the pathogenicity of isolates with these mutations on cucumber leaves were very similar to that of the other *BenA* mutants or the wild-type strain (Fig. 2). The fungicide programs for gray mold control have been shifted from the use of mixtures of diethofencarb and carbendazim or thiophanate-methyl to new classes of fungicides such as phenylpyrroles, anilinoimidazoles, and hydroxylanilides. The isolates bearing the E198K or F200Y mutations might be less fit than the wild-type strains or resistant isolates with the E198A or E198V mutation in the absence of fungicide selection.

We previously reported that dicarboximide-resistant strains with the *BcOS1* I365S mutation are widely spread throughout Japan, and the other two types of dicarboximide-resistant mutations were detected among isolates in the Osaka area collected in 2000; one isolate has three amino acid substitutions (V368F, Q369H, plus T447S) and the other isolate has two amino acid substitutions (Q369P plus N373S) within the histidine kinase *BcOS1p* (Table 5). The *BcOS1* genotyping of field isolates collected from 2002 to 2005 revealed that these two types of dicarboximide-resistant strains were established in the Osaka area. Ma et al. (19) reported that some dicarboximide-resistant strains isolated in California carried the Q369P mutation in the *BcOS1* gene; this may suggest the global distribution of this mutation. Other types of dicarboximide-resistant mutations in the *BcOS1* gene that cause a single amino acid substitution (I365N or I365R) instead of I365S were found in New Zealand, Europe, and the United States (5,15,19). Additionally, a strain with two amino acid substitutions (i.e., I365S and Q369P) was found in Pukekohe, New Zealand (5). We synthesized three single-stranded oligonucleotides whose nucleotide sequences matched either those of the *BcOS1* I365N, I365R, or I365S plus Q369P mutations and

TABLE 5. Genotyping of benzimidazole- and dicarboximide-resistant isolates of *Botrytis cinerea* from Osaka fields in 2000, 2002–2003, and 2004–2005

Year(s)/ <i>BcOS1</i> genotype	Number of isolates with <i>BenA</i> mutation					Total
	Wild type	E198A	F200Y	E198K	E198V	
2000						
Wild type	2	2	0	0	1	5
I365S	0	2	0	0	10	12
V368F, Q369H	8	2	0	0	0	10
Q369P	0	2	0	0	0	2
Total	10	8	0	0	11	29
2002–2003						
Wild type	9	5	0	0	3	17
I365S	0	5	0	0	5	10
V368F, Q369H	5	0	0	0	0	5
Q369P	0	0	0	0	8	8
Total	14	10	0	0	16	40
2004–2005						
Wild type	18	5	0	0	6	29
I365S	0	0	1	0	19	20
V368F, Q369H	3	1	0	0	0	4
Q369P	0	0	0	0	12	12
Total	21	6	1	0	37	65

were used as a template for thermal analysis to obtain the melting curve profiles of these mutants. The peak temperatures of these dicarboximide-resistant mutants did not match any of those of the four types of Japanese isolates used in this study (data not shown). Therefore, we conclude that all the isolates we collected from the Japanese fields belong to one of four types regarding the *BcOS1* gene. Recently, a new dicarboximide-resistant *BcOS1* mutation which causes immature termination at amino acid position 1040 of BcOS1p was found in one isolate in the United States (19), yet obviously our protocol is not designed to detect this mutation.

The hybridization assays developed enable the classification of *B. cinerea* into a total of 20 types (Tables 4 and 5). Genotyping analyses of the *BenA* and *BcOS1* genes provide important information for the selection of fungicides for the control of gray mold. First, a mixture of a benzimidazole fungicide, carbendazim or thiophanate-methyl, with diethofencarb controls the wild-type isolates and isolates with the E198A and probably the E198V mutations in the *BenA* gene but not those with the E198K or F200Y mutation (Table 3). Second, dicarboximide-resistant isolates frequently carry the benzimidazole-resistant mutation, particularly in isolates with the *BcOS1* I365S mutation. In other words, only a small portion of the isolates were sensitive to benzimidazoles and resistant to dicarboximides. Similar results were reported for *B. cinerea* collected in South Carolina (28,29). These observations suggested that not only a particular mutation in *BenA* and *BcOS1* but also their combination may affect the fitness and stability of *B. cinerea* isolates in the field.

The hybridization assay based on PCR amplification and thermal analysis is a reliable detection procedure that does not involve postamplification steps such as restriction enzyme digestion and electrophoresis of the DNA fragment. This method based on the use of a fluorescent hybridization probe in real-time PCR has been successfully applied in the mutational analysis of several drug-resistant clinical pathogens (4,16,24). In conclusion, we emphasize that such hybridization assays could be useful for the detection of mutations resistant to other fungicides in various pathogens with clear advantages. The commonly used PCR-based method specifically detects only one type of mutation; however, the dissociation curve analysis in the hybridization probe assay may have the potential to detect any mutation within the annealing region of the probes. Therefore, this method would be applicable for the detection of mutations resistant to new fungicides such as the mitochondria complex III inhibitor (quinone outside inhibitors) in various pathogens.

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