Molecular Characterization of Boscalid Resistance in Field Isolates of *Botrytis cinerea* from Apple

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ABSTRACT

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Botrytis cinerea isolates obtained from apple orchards were screened for resistance to boscalid. Boscalid-resistant (Bos^R) isolates were classified into four phenotypes based on the levels of the concentration that inhibited fungal growth by 50% relative to control. Of the 220 isolates tested, 42 were resistant to boscalid, with resistant phenotypes ranging from low to very high resistance. There was cross resistance between boscalid and carboxin. Analysis of partial sequences of the iron-sulfur subunit of succinate dehydrogenase gene in *B. cinerea* (*BcSdhB*) from 13 Bos^R and 9 boscalid-sensitive (Bos^S) isolates showed that point mutations in *BcSdhB* leading to amino acid substitutions at the codon position 272 from histidine to either tyrosine (H272Y) or arginine (H272R) were correlated with boscalid resistance. Allele-specific polymerase chain reaction (PCR) analysis of 66 Bos^R isolates (including 24 additional isolates obtained from decayed apple fruit) showed that 19 carried the point mutation H272Y and 46 had the point mutation H272R,

but 1 Bos^R isolate gave no amplification product. Analysis of the *BcSdhB* sequence of this isolate revealed a different point mutation at codon 225, resulting in a substitution of proline (P) by phenylalanine (F) (P225F). The results indicated that H272R/Y in *BcSdhB* were the dominant genotypes of mutants in field Bos^R isolates from apple. A multiplex allele-specific PCR assay was developed to detect point mutations H272R/Y in a single PCR amplification. Levels of boscalid resistance ranged from low to very high within isolates carrying either the H272R or H272Y mutation, indicating that, among Bos^R isolates, different Bos^R phenotypes (levels of resistance) were not associated with particular types of point mutations (H272R versus H272Y) in *BcSdhB*. Analysis of genetic relationships between 39 Bos^R and 56 Bos^S isolates based on three microsatellite markers showed that 39 Bos^R isolates and 30 Bos^S isolates were clustered into two groups, and the third group consisted of only Bos^S isolates, suggesting that the development of resistance to boscalid in *B. cinerea* likely is not totally random, and resistant populations may come from specific genetic groups.

Additional keywords: carboxamide, fungicide resistance, SDHI fungicides.

Botrytis cinerea Pers. (teleomorph *Botryotinia fuckeliana* (de Bary) Whetzel) causes pre- and postharvest diseases in many economically important crops, including vegetable, fruit, and ornamental plants (17). Synthetic fungicides remain the major tools for control of *Botrytis cinerea* (21). However, *B. cinerea* is a high-risk pathogen for the development of fungicide resistance because of its short life cycle and prolific reproduction (6). Resistance of *B. cinerea* to fungicides negatively affects control efficacy and often results in the failure of disease control (21).

Gray mold caused by *B. cinerea* is a major postharvest fruit rot disease of apple. Control of gray mold in stored apple fruit can be achieved by applying fungicides either near harvest in the orchard or after harvest as a postharvest treatment (30). The boscalid-containing product Pristine (BASF Corporation, Research Triangle Park, NC), a premixed formulation of boscalid and pyraclostrobin, was registered in 2005 for use on apple in the United States. Application of Pristine within 2 weeks before harvest is effective in controlling gray mold in stored apple fruit, and this practice is being increasingly implemented since 2005 by apple growers and packers for control of gray mold and other postharvest diseases in stored apple fruit (30). However, resistance to boscalid was observed in *B. cinerea* isolates obtained from gray mold-decayed, stored apple fruit originating from apple orchards where Pristine had been used in each of the four consecutive

years since 2005 (19). Although the vast majority of boscalid-resistant (Bos^R) isolates obtained from stored apple fruit also are resistant to pyraclostrobin, there is no cross resistance between boscalid and pyraclostrobin, suggesting that boscalid resistance is a result of selection pressure from the use of the boscalid-containing fungicide Pristine in apple orchards (19). However, there is no information available about the frequency of Bos^R isolates in apple orchards where the fungicide had been used (19).

Boscalid is a recently introduced fungicide belonging to the class of succinate dehydrogenase inhibitors (SDHIs). In addition to boscalid, SDHIs also include other compounds such as carboxin and flutolanil (10), which have similar chemical structures and modes of action (16,28). The target site of SDHIs is succinate ubiquinone reductase or succinate dehydrogenase (SDH) complex, which is a functional part of the tricarboxylic acid cycle and the mitochondrial electron transport chain (18). The SDH complex consists of four subunits; that is, a flavoprotein (Fp) subunit (SdhA); an iron-sulfur protein (Ip) subunit (SdhB), including three iron-sulfur clusters; and two membrane-anchored protein subunits (SdhC and SdhD) (11,16). Previous studies have documented that the SDH binding site is composed of the residues in subunits of SdhB, SdhC, and SdhD and is highly conserved in bacteria and eukaryotes (13,14,29).

Substitutions of conserved amino acids in the SDH complex conferring resistance to SDHI fungicides have been reported in various microorganisms (2–4,7,12,15,16,26–28). In most cases, single-point mutations correlating with SDHI resistance commonly occur on a highly conserved histidine residue located in the third cysteine-rich cluster in the SdhB subunit. In *B. cinerea*,

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laboratory Bos^R mutants can be readily obtained by UV mutagenesis (8,28,31). Molecular analyses based on lab mutants and field-resistant isolates indicated mutations at the amino acid positions of 272 (H272Y/R) and 225 (P225L/F/T) in *BcSdhB* correlating with boscalid resistance (8,28). More recently, Leroux et al. (22) reported that, in addition to mutations H272Y/R, single-point mutations H272L and N230I in *BcSdhB* and H132R in *BcSdhD* were also correlated with boscalid resistance in *B. cinerea* isolates obtained from infected berries in France and Germany; however, the latter three mutations were much less common in the field. Stammler (28) reported that mutations H272Y and P225L/F in the *BcSdhB* gene were associated with significantly reduced sensitivities to boscalid in *B. cinerea*, whereas the reduction in sensitivity was less pronounced in isolates carrying mutations H272R and P225T. However, no detailed information, such as frequency of resistant phenotypes and genotypes as well as their ranges of the concentration that inhibits fungal growth by 50% relative to control (EC₅₀ values), was provided in the report (28). It remains to be determined whether different phenotypes of boscalid resistance (levels of resistance) are associated with particular types of point mutations in *BcSdhB* among Bos^R isolates of *B. cinerea*.

The objectives of this study were to (i) assess the frequency of Bos^R isolates in *B. cinerea* populations collected from apple orchards where the fungicide had been used, (ii) determine whether there is cross-resistance between boscalid and carboxin, (iii) characterize mutations in the SdhB subunit of SDH correlating with boscalid resistance and determine whether Bos^R phenotypes (levels of resistance) in field-resistant isolates of *B. cinerea* are associated with particular types of point mutations in the *BcSdhB* gene, (iv) develop polymerase chain reaction (PCR) assays for molecular diagnosis of boscalid resistance, and (v) assess genetic relationships between Bos^R and boscalid-sensitive (Bos^S) isolates of *B. cinerea* from apple.

MATERIALS AND METHODS

Frequency of boscalid resistance in apple orchards treated with Pristine. To determine frequency of Bos^R *B. cinerea* isolates in apple orchards, 'Gala' apple fruit were sampled in August and September 2009 before harvest from five orchards located in Brewster, WA. In these orchards, the boscalid-containing fungicide Pristine had been used in each of the four consecutive years since 2005, and boscalid resistance had been detected in *B. cinerea* isolates recovered from decayed apple fruit originating from the 2008 crops from these orchards (19). In each orchard, 102 symptomless Gala apple fruit were hand picked from 34 trees (3 fruit per tree). To isolate *B. cinerea* from the fruit, the stem and floral parts of each fruit were excised, briefly rinsed with sterile water, and air dried on sterilized paper in a laminar-flow hood. Tissues were placed on acidified potato dextrose agar (APDA) (Difco Laboratories, Detroit) (4 ml of a 25% lactic acid per liter of medium) in petri dishes. Plates were incubated at 20°C in the

dark and examined for culture development. Fungal colonies similar to those of *B. cinerea* on isolation plates were transferred onto fresh APDA. Pure cultures were then grown on PDA and inoculated at 20°C under 12 h of light and 12 h of darkness to facilitate sporulation. *B. cinerea* isolates were then single-spore cultured and mycelium plugs were stored in 15% glycerol at -80°C. In total, 220 single-spore isolates of *B. cinerea* were obtained from these five orchards (Table 1).

Technical-grade boscalid (99% a.i.) (BASF Corporation) was dissolved in 100% acetone to provide stock solutions containing 25 mg/ml. Sensitivity of conidial germination to boscalid was tested on 2% water agar (WA) amended with boscalid at the minimum inhibitory concentration (MIC) level (5 µg/ml) following the procedure of Kim and Xiao (19). Isolates that were able to germinate were considered resistant to boscalid, whereas those that were not able to germinate were classified as Bos^S isolates.

Phenotypes of Bos^R isolates. In total, 172 (66 Bos^R and 106 Bos^S) *B. cinerea* isolates were included in this study. Of these isolates, 42 Bos^R (Table 2) and 54 Bos^S isolates came from the 220 single-spore isolates obtained in 2009 from five apple orchards as described above. The remaining 24 Bos^R and 52 Bos^S isolates were obtained during 2001 to 2009 from decayed apple fruit originating from orchards in the main apple-production areas in central Washington (19). All isolates used in this study were single-spore cultured.

For all Bos^R isolates, EC₅₀ values of boscalid were determined in a conidial germination assay following the procedure of Kim and Xiao (19), and the experiment was performed twice. Stock solutions of technical-grade boscalid or a formulated boscalid (Endura 70% a.i.; BASF Corporation) were added into 2% WA to produce concentrations of boscalid in the medium of 0, 1, 2.5, 5, 10, 20, 63.1, 189.3, and 631 µg/ml. The formulated product was used to make higher concentrations of boscalid (>20 µg/ml) in the medium because of limited solubility of technical-grade boscalid in acetone (19). In the boscalid baseline sensitivity study for *B. cinerea* populations from apple in Washington State, the mean EC₅₀ value of boscalid was 0.631 µg/ml for Bos^S isolates (19). In the present study, Bos^R isolates were further classified into various resistance phenotypes based on the EC₅₀ values of boscalid for them: low resistance to boscalid if the resistance factor (RF) (folds of EC₅₀ value of boscalid for a resistant isolate relative to the mean EC₅₀ value of boscalid [= 0.631 µg/ml] for the baseline sensitive isolates) < 10, moderate resistance if 10 ≤ RF < 100, high resistance if 100 ≤ RF < 1,000, and very high resistance if RF ≥ 1,000.

Cross-resistance analysis between boscalid and carboxin. In all, 13 Bos^R isolates representing different levels of boscalid resistance and 6 Bos^S isolates were examined for cross-resistance between boscalid and carboxin (Table 3). A formulated carboxin (Vitavax, 34% a.i.) (Bayer CropScience, Research Triangle Park, NC) was dissolved in sterile distilled water to provide a stock solution at a concentration of 20 µg/ml. Carboxin stock solution was added into 2% WA to produce the following concentrations:

TABLE 1. Boscalid resistance in *Botrytis cinerea* isolates obtained from the stem and floral parts of symptomless 'Gala' apple fruit in commercial orchards

Orchard ^a	Number of isolates	Number of Bos ^R isolates ^b	Number of isolates carrying mutation in the <i>BcSdhB</i> gene ^c		
			H272Y	H272R	P225F
C1	51	19	1	17	1
C2	45	9	4	5	0
C3	24	3	1	2	0
C4	47	5	2	3	0
C5	53	6	3	3	0
Total	220	42	11	30	1

^a Pyraclostrobin + boscalid (Pristine) had been used in each of the four consecutive years in these orchards since 2005.

^b Bos^R = boscalid resistant. Resistance phenotypes were determined at a discriminatory concentration of 5 µg/ml on water agar in a conidial germination assay (19). Conidial germination of sensitive isolates was completely inhibited at the discriminatory concentration, whereas resistant isolates were able to germinate.

^c H272Y and H272R = the amino acid His replaced respectively by Tyr and Arg at codon 272; P225F = Pro substituted by Phe at codon 225.

TABLE 2. Characteristics of boscalid-resistant isolates of *Botrytis cinerea* from apple

Isolate	Year of isolation	Orchard ^a	Type of sample ^b	EC ₅₀ of boscalid	Phenotype ^c	Mutation in <i>BcSdhB</i>	Type of mutant ^d
6632	2007	O1	A	8.82	MR	H272R	II
7420	2009	O1	A	86.608	HR	H272R	II
7434	2009	O1	A	251.74	HR	H272R	II
7436	2009	O1	A	63.350	HR	H272R	II
7437	2009	O1	A	17.441	MR	H272Y	I
7391	2008	C8	A	490.25	HR	H272Y	I
7392	2008	C8	A	17.517	MR	H272R	II
7451	2009	C4	A	12.122	MR	H272R	II
7453	2009	C1	A	357.103	HR	H272R	II
7455	2009	C3	A	9.363	MR	H272R	II
7465	2009	C5	A	>631	VHR	H272Y	I
7466	2009	C5	A	>631	VHR	H272Y	I
7474	2009	C3	A	6.385	MR	H272R	II
7477	2009	C3	A	6.421	MR	H272R	II
7491	2009	C3	A	>631	VHR	H272R	II
7494	2009	C2	A	7.197	MR	H272R	II
7626	2009	C7	A	5.850	LR	H272Y	I
7633	2009	C7	A	237.877	HR	H272R	II
7635	2009	C7	A	414.594	HR	H272R	II
7636	2009	C7	A	9.029	MR	H272R	II
7638	2009	C7	A	13.386	MR	H272Y	I
7640	2009	C7	A	>631	VHR	H272Y	I
7647	2009	C7	A	6.122	LR	H272Y	I
7679	2009	C6	A	5.799	LR	H272R	II
7936	2009	C2	B	8.759	MR	H272R	II
7999	2009	C2	B	4.473	LR	H272Y	I
8000	2009	C2	B	214.989	HR	H272R	II
8001	2009	C2	B	>631	VHR	H272Y	I
8002	2009	C2	B	159.583	HR	H272Y	I
8003	2009	C2	B	10.155	MR	H272R	II
8016	2009	C2	B	9.288	MR	H272Y	I
8018	2009	C2	B	17.057	MR	H272R	II
8019	2009	C2	B	12.117	MR	H272R	II
8022	2009	C5	B	73.698	HR	H272R	II
8036	2009	C5	B	>631	VHR	H272Y	I
8080	2009	C5	B	>631	VHR	H272Y	I
8093	2009	C4	B	8.297	MR	H272R	II
8098	2009	C4	B	18.384	MR	H272R	II
8122	2009	C4	B	10.454	MR	H272R	II
8133	2009	C4	B	4.601	LR	H272Y	I
8144	2009	C4	B	7.511	MR	H272Y	I
8170	2009	C3	B	7.375	MR	H272R	II
8178	2009	C3	B	177.96	HR	H272R	II
8184	2009	C3	B	>631	VHR	H272Y	I
8186	2009	C1	B	31.462	MR	H272R	II
8193	2009	C1	B	11.485	MR	H272R	II
8195	2009	C1	B	4.732	LR	H272R	II
8196	2009	C1	B	8.032	MR	H272R	II
8198	2009	C1	B	12.156	MR	H272R	II
8200	2009	C1	B	>631	VHR	H272R	II
8201	2009	C1	B	4.445	LR	H272R	II
8204	2009	C1	B	8.848	MR	H272R	II
8208	2009	C1	B	108.836	HR	H272R	II
8209	2009	C1	B	>631	VHR	H272R	II
8211	2009	C1	B	6.162	LR	H272R	II
8218	2009	C1	B	8.094	MR	H272R	II
8219	2009	C1	B	6.469	MR	H272R	II
8220	2009	C1	B	>631	VHR	P225F	III
8222	2009	C1	B	7.474	MR	H272R	II
8223	2009	C1	B	6.215	LR	H272R	II
8230	2009	C1	B	6.294	LR	H272R	II
8232	2009	C1	B	9.221	MR	H272Y	I
8234	2009	C1	B	7.235	MR	H272R	II
8255	2009	C5	B	>631	VHR	H272Y	I
8300	2009	C5	B	>631	VHR	H272R	II
8301	2009	C5	B	16.137	MR	H272R	II

^a In conventional orchards (C1 to C8), Pristine (pyraclostrobin + boscalid) had been used in each of the four consecutive seasons since 2005, whereas no synthetic fungicides were used in the organic orchard (O1) since the mid-1990s.

^b A = decayed fruit sampled from fruit packinghouses and B = stems and floral parts of symptomless fruit sampled from apple orchards before harvest.

^c LR = low resistance to boscalid (resistance factor [RF] < 10), MR = moderate resistance to boscalid (10 ≤ RF < 100), HR = high resistance to boscalid (100 ≤ RF < 1,000), and VHR = very high resistance to boscalid (RF ≥ 1,000).

^d I, II, and III = boscalid-resistant mutants carrying H272Y, H272R, and P225F mutations, respectively, in the *BcSdhB* gene.

0, 0.1, 0.5, 1, 2.5, 5, 7.5, 10, and 20 µg/ml for Bos^R isolates and 0, 0.01, 0.05, 0.1, 0.25, 0.5, and 0.75 µg/ml for Bos^S isolates. EC₅₀ values for conidial germination were determined as described above. The experiment was performed twice.

Correlation analysis was performed using PROC CORR of SAS ($P = 0.05$) to determine whether there was cross resistance between boscalid and carboxin for 14 isolates tested. The log-transformed EC₅₀ values of both fungicides were used for analysis. The five very highly Bos^R isolates were not included in the analysis because no definitive EC₅₀ values of boscalid (>631 µg/ml) were available for these isolates. However, a t test using SAS PROC TTEST ($P = 0.05$) was performed with all isolates listed in Table 3 to determine whether EC₅₀ values of carboxin for Bos^R isolates were significantly higher than those for Bos^S isolates.

Analysis of DNA sequence of the *BcSdhB* gene from Bos^R and Bos^S isolates. Genomic DNA was extracted from mycelium of *B. cinerea* using the FastDNA kit and the FastPrep instrument (MP Biomedicals, Irvine, CA) according to the manufacturer's

instructions. Based on the sequence of the *SdhB* gene in *B. cinerea* (*BcSdhB*) (GenBank accession number AY726618.1), the primer pair SdhB-F + SdhB-R (Table 4) was designed to amplify a fragment containing the codons 225 and 272 in the *BcSdhB* gene from 13 Bos^R and 9 Bos^S isolates. PCR amplifications were performed in a 25-µl volume containing 20 ng of fungal genomic DNA, 0.2 µM each primer, 0.2 mM each of dNTP (Promega Corp., Madison, WI), 1.5 mM MgCl₂, 1× Promega Taq polymerase buffer (10 mM Tris-HCl, pH 9.0; 50 mM KCl; and 0.1% Triton X-100), and 1.5 units of Taq polymerase (Promega Corp.). PCR was carried out in an iCycler thermal cycler (Bio-Rad Life Science Research Group, Hercules, CA) with the following parameters: an initial preheating for 3 min at 95°C; followed by 35 cycles of denaturation at 94°C for 40 s, annealing at 56°C for 40 s, and extension at 72°C for 1 min; and a final extension at 72°C for 5 min. The PCR products were separated on 1.5% agarose gels in 1× Tris-acetate-EDTA (TAE) buffer and photographed after staining with ethidium bromide.

Using this pair of primers, a 675-bp single PCR fragment was amplified from each tested isolate. PCR products were purified using the QIAquick PCR purification kit (Qiagen Inc., Valencia, CA) according to the manufacturer's instructions. Cycle sequencing was performed using the ABI Prism BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA) and PCR primers. The sequencing reaction mixture contained 0.5 to 1 µg of DNA, 3.2 pmol primer, 4 µl of BigDye Terminator Cycle Sequencing Ready Reaction Mix, and sterile distilled water in 10-µl total volumes. Cycle sequencing was carried out at 96°C for 15 s, 50°C for 15 s, and 60°C for 4 min for 25 cycles. Products were purified using Centriflex Gel Filtration Cartridges (Edge Biosystems, Gaithersburg, MD) and dried in a speed vacuum concentrator. Sequences were read in the Laboratory for Biotechnology and Bioanalysis, School of Molecular Biosciences, Washington State University, Pullman. Representative sequences were deposited in GenBank under accession numbers HQ622627 to HQ622630.

Development of allele-specific PCR assays for detection of mutations at the codon position 272 in the *BcSdhB* gene. Two types of point mutations at the codon position 272 (H272Y/R) in *BcSdhB* were observed in Bos^R isolates sequenced, and we then designed three pairs of allele-specific primers for detection of these point mutations (Table 4). Based on one point mutation from C (CAC in Bos^S isolates) to T (TAC in Bos^R isolates) at the codon position 272, a nucleotide A at the 3' end of the reverse primer 272Y-R was designed to match the T at this codon position in Bos^R isolates. Similarly, based on the other point mutation from A to G (CGC in Bos^R isolates) at the codon position 272, a nucleotide G at the 3' end of the forward primer 272R-F2 and a nucleotide C at the 3' end of the reverse primer 272R-R were designed to match the G at this codon position in Bos^R isolates. Previous studies have shown that the addition of an extra mismatch base, together with the natural mutation base at the 3' end of primer, can increase the specificity of the primers in

TABLE 3. Sensitivity of conidial germination to carboxin in boscalid-sensitive and -resistant isolates of *Botrytis cinerea* from apple

Isolate	Phenotype ^a	Genotype ^b	EC ₅₀ (µg/ml) ^c	
			Boscalid	Carboxin
1927	S	Wild	0.919	0.120
1922	S	Wild	1.476	0.145
1940	S	Wild	0.586	0.125
1859	S	Wild	0.667	0.104
6631	S	Wild	0.341	0.055
6705	S	Wild	0.911	0.070
Mean	...	...	0.817	0.103
7999	LR	I	4.473	0.459
8133	LR	I	5.580	1.750
7638	MR	I	17.441	3.340
8184	VHR	I	>631	2.350
8080	VHR	I	>631	1.683
7640	VHR	I	>631	1.563
8201	LR	II	4.445	0.595
7679	LR	II	5.799	0.752
8186	MR	II	31.462	1.011
7420	HR	II	86.608	1.325
7633	HR	II	237.877	2.625
8300	VHR	II	>631	2.183
8220	VHR	III	>631	3.848
Mean	...	...	>272.976	1.806

^a Phenotype of sensitivity to boscalid: S = sensitive to boscalid, LR = low resistance to boscalid (resistance factor [RF] < 10), MR = moderate resistance to boscalid (10 ≤ RF < 100), HR = high resistance to boscalid (100 ≤ RF < 1,000), VHR = very high resistance to boscalid (RF ≥ 1,000).

^b Wild = wild type, I = boscalid-resistant mutants carrying mutation H272Y in the *BcSdhB* gene, II = boscalid-resistant mutants carrying mutation H272R in the *BcSdhB* gene, and III = boscalid-resistant mutants carrying mutation P225F in the *BcSdhB* gene.

^c EC₅₀ values = the concentration of boscalid that inhibits fungal growth by 50% relative to control for the six boscalid-sensitive isolates originated from the previous study reported by Kim and Xiao (19).

TABLE 4. Polymerase chain reaction (PCR) primers used in this study

Primer	Sequence (5'–3')	Relevant characteristics ^a
SdhB-F	AAGGTATCTGCGGCAGTTGTG	PCR primers for amplification of partial <i>SdhB</i> gene containing codons 225 and 272 in <i>Botrytis cinerea</i>
SdhB-R	TAGCAATAACCGCCCAAAAC	
272Y/R-F	AAGGAGGATCGTAAGAAGCTTG	Allele-specific PCR primers for the detection of Bos ^R isolates of <i>B. cinerea</i> carrying the H272Y mutation
272Y-R	TCGAGCAGTTGAGAATAGTGTA	
272Y/R-F	AAGGAGGATCGTAAGAAGCTTG	Allele-specific PCR primers for the detection of Bos ^R isolates of <i>B. cinerea</i> carrying the H272R mutation
272R-R	TCGAGCAGTTGAGAATAGTGC	
272R-F2	GCATGAGTTGTACAGATGTAG	...
272R-R2	TTAGCAATAACCGCCCAAAAC	
(AAC) ₅	AACAACAACAACAAC	Microsatellite primers for analysis of genetic variation in populations of <i>B. cinerea</i>
(GACA) ₄	GACAGACAGACAGACA	
M13	GAGGGTGGCGGTTCT	

^a Bos^R = boscalid resistant.

detecting point mutations (9,32). To improve the specificity of traditional allele-specific PCR amplifications, we introduced an artificial mismatch base A at the second nucleotide at the 3' end of primer 272R-F2 (Table 4). Primers 272Y/R-F and 272R-R2 were designed based on the sequence AY726618.1 in GenBank. PCR amplification parameters were an initial preheating for 3 min at 95°C; 35 cycles of denaturation at 94°C for 30 s, annealing at 62°C (272Y/R-F + 272Y-R or 272R-R), 59°C (272R-F2 + 272R-R2) for 30 s, and extension at 72°C for 30 s; and terminated with a final extension at 72°C for 5 min.

All 66 Bos^R and 106 Bos^S isolates were tested for the specificity of three allele-specific PCR assays described above. Amplifying with the three pairs of primers (272Y/R-F + 272Y-R, 272Y/R-F + 272R-R, and 272R-F2 + 272R-R2) (Table 4), 65 Bos^R isolates gave positive amplifications, and 106 Bos^S isolates and 1 Bos^R isolate 8022 produced no PCR amplifications. DNA sequencing was further performed for isolate 8022 to detect whether other point mutations occurred in the *BcSdhB* gene with primer pair SdhB-F + SdhB-R.

For the simultaneous detection of both mutations at the codon position 272 in *BcSdhB*, three primers (272Y/R-F, 272Y-R, and 272R-R) were used in single-PCR amplifications. PCR conditions used for the multiplex PCR assay were the same as described above.

Genetic relationships between the Bos^R and Bos^S isolates. To investigate genetic relationships between the Bos^R and Bos^S isolates, 39 Bos^R and 56 Bos^S isolates were analyzed by microsatellite-primed PCR (MP-PCR) with three microsatellite primers: (AAC)₅, (GACA)₄, and M13 (Table 4). These three primers were chosen because they were informative in determining genetic relationships among *B. cinerea* isolates (24). PCR was conducted with an initial preheat at 95°C for 3 min; followed by 40 cycles of denaturation at 94°C for 1 min, annealing at 45°C for 1 min, and extension at 72°C for 1.5 min; and terminated with a final extension at 72°C for 7 min. Each isolate was amplified twice. The PCR products were separated on 1.2% agarose gels in 1× TAE buffer and photographed after staining with ethidium bromide.

To analyze MP-PCR data, each isolate was scored for the presence or absence of each amplicon using UVP GelDoc-It Imaging System (Ultra Violet Products, Upland, CA). Genetic similarities (S) were calculated using simple matching coefficient. A phenogram was constructed using the unweighted pairgroup method with arithmetic average (UPGMA) by the program Sequential, Agglomerative, Hierarchical, and Nested clustering methods of the software package NTSYS-pc 2.1 (Department of Ecology and Evolution, State University of New York).

RESULTS

Boscalid resistance in apple orchards. Of the 220 isolates obtained from Gala apple fruit in five orchards where the boscalid-containing fungicide Pristine had been used for at least four consecutive years, 42 isolates (19.1%) were resistant to boscalid (Table 1). The frequency of Bos^R isolates was 11 to 37% among the orchards sampled.

Phenotypes of boscalid resistance. EC₅₀ values of the 42 Bos^R isolates obtained from apple fruit in orchards and 24 additional Bos^R isolates obtained from decayed apple fruit collected from commercial fruit packinghouses ranged from 4.445 to >631 µg/ml and from 5.799 to >631 µg/ml, respectively (Table 2). Of the 66 Bos^R isolates, 10 were low resistant to boscalid, with EC₅₀ values of 4.445 to 6.294 µg/ml (RF < 10); 31 were moderately resistant, with EC₅₀ values of 6.385 to 31.462 µg/ml (10 < RF < 100); 12 were highly resistant, with EC₅₀ values of 63.35 to 490.25 µg/ml (100 < RF < 1,000); and 13 were very highly resistant, with EC₅₀ values >631 µg/ml (RF > 1,000) (Table 2).

Cross-resistance between boscalid and carboxin in *B. cinerea*. The EC₅₀ values of carboxin for 13 Bos^R and 6 Bos^S

isolates are shown in Table 3. Pearson correlation analysis indicated that EC₅₀ values between boscalid and carboxin were highly correlated ($r = 0.8853$, $P < 0.0001$, $n = 14$). The t test also showed that the mean EC₅₀ values of carboxin for Bos^R isolates were significantly higher than those for Bos^S isolates ($P < 0.0001$, $n = 19$), indicating a high level of cross-resistance between boscalid and carboxin.

Sequence comparison of the *BcSdhB* gene between Bos^R and Bos^S isolates. The partial nucleotide sequences of *BcSdhB* were compared for 13 Bos^R isolates (6632, 7420, 7434, 7436, 7437, 7391, 7392, 7451, 7453, 7455, 7465, 7466, and 7494) and 9 Bos^S isolates (288, 1753, 1760, 1924, 6627, 6628, 6635, 7435, and 7452). Analysis of the DNA sequence of *BcSdhB* showed that the codon CAC for His at the position 272 was replaced by the codon TAC for Tyr in four Bos^R isolates (H272Y), and by CGC for Arg in nine Bos^R isolates (H272R) (Fig. 1). Except for the two point mutations at position 272 in Bos^R isolates, all 22 isolates analyzed had identical deduced amino acid sequences in the *BcSdhB* gene.

Molecular diagnosis of Bos^R isolates mutated in the *BcSdhB* gene. The expected 232-bp fragment was amplified from Bos^R isolates carrying mutations H272Y or H272R in the *BcSdhB* gene using the primer pairs 272Y/R-F + 272Y-R and 272Y/R-F + 272R-R, respectively. No amplifications were observed from the 106 Bos^S isolates tested and 1 Bos^R isolate carrying mutations other than H272Y/R in the *BcSdhB* gene (Fig. 2A and B). Using primer pair 272R-F2 + 272R-R2, the expected 197-bp fragment was amplified only from Bos^R isolates carrying the mutation H272R (data not shown). Of the 42 field Bos^R isolates from five orchards, 11 had the mutation H272Y (designated as type I mutant) and 30 carried the mutation H272R (designated as type II mutant) (Tables 1 and 2), indicating that type I and II mutants were the major genotypes of Bos^R populations in the field. Among the total 66 Bos^R isolates tested (including 24 Bos^R isolates obtained from decayed apple fruit), 19 belonged to the type I mutant and 46 belonged to the type II mutant (Table 2). Analyzing the sequence of the *BcSdhB* gene for isolate 8220 that gave no amplifications with the three pairs of primers described above (Fig. 2A and B), a new point mutation (P225F) was detected. The codon CCC for Pro (P) at position 225 present in the Bos^S isolates was changed to the TTC for Phe (F) in isolate 8220. The mutation P225F was designated as a type III mutant (Fig. 1; Table 2).

To simultaneously detect both mutations at codon 272 in single-PCR amplifications, a multiplex PCR assay was developed. Analysis of 66 Bos^R and 106 Bos^S isolates with the multiplex PCR assay showed that a 232-bp fragment was amplified from each of the 65 Bos^R isolates carrying either the H272Y or H272R mutation, and that no PCR amplifications were obtained from the type III mutant containing mutation P225F and all Bos^S isolates (Fig. 2C).

Levels of boscalid resistance within Bos^R isolates carrying either the H272R or H272Y mutation. Of the 19 type I mutants carrying the H272Y mutation, 4 (21.1%) exhibited a low level of resistance to boscalid, with RF < 10; 5 (26.3%) were moderately resistant, with RF of 10 to 100; 2 (10.5%) were highly resistant, with RF of 100 to 1,000; and 8 (42.1%) were very highly resistant, with RF > 1,000 (Fig. 3). Of the 46 type II mutants containing the H272R mutation, 6 (13.0%) showed low resistance to boscalid, 26 (56.5%) were moderately resistant, 10 (21.7%) were highly resistant, and 4 (8.7%) were very highly resistant (Fig. 3). The results indicated that all four Bos^R phenotypes were present in both type I and type II mutants and that different Bos^R phenotypes were not associated with particular types of mutations at codon position 272 among Bos^R isolates.

Genetic relationships between Bos^R (type I and II mutants) and Bos^S *B. cinerea* isolates. Three microsatellite primers generated a total of 27 polymorphic fragments and 46 haplotypes

from 39 Bos^R (19 type I, 19 type II, and 1 type III mutants) and 56 Bos^S isolates. Examples of DNA fingerprint patterns generated by the primer (AAC)₅ are presented in Figure 4. The UPGMA analysis showed that 97.9% isolates were clustered into groups A (41.1%), B (31.6%), and C (25.3%) (Fig. 5). In all, 28 Bos^R (15 type I, 12 type II, and 1 type III mutants) and 11 Bos^S isolates were clustered into group A. In total, 11 Bos^R (4 type I and 7 type II mutants) and 19 Bos^S isolates were clustered into group B. Group C consisted of 24 Bos^S isolates, and no Bos^R isolates were clustered into this group (Fig. 5). Additionally, there was no specific association between mutation types and genetic groups based on the MP-PCR analysis among Bos^R mutants.

DISCUSSION

In the present study, we found a high frequency of Bos^R isolates of *B. cinerea* in apple orchards where the boscalid-containing fungicide Pristine had been used for four consecutive years. Given the fact that Pristine was used only once a year and no other boscalid-containing fungicides were used in these orchards, the results suggest that the risk for development of boscalid resistance in *B. cinerea* is considered high. In a previous related study, boscalid resistance was observed in *B. cinerea* isolates obtained from stored, gray mold-decayed apple fruit sampled from packinghouses, and ≈20% of the isolates were resistant to boscalid (19). In that study, decayed apple fruit originated from the

previous season's crops grown in the same orchards whereas, in the present study, *B. cinerea* isolates were collected near harvest before the fungicide Pristine was applied in these apple orchards. Thus, no boscalid-containing fungicides had been applied in these orchards between the two collections. It remains to be determined whether resistance frequency will increase if the boscalid-containing Pristine continues to be used in these orchards.

Numerous studies on the molecular mechanisms conferring SDHI resistance in mutants of various fungi and bacteria have shown that mutations in highly conserved amino acid residues located in subunits SdhB, SdhC, or SdhD in SDH responsible for SDHI resistance (3,4,7,12,15,16,18,23,26–28). It appears that amino acid positions at which mutations occur and types of mutations vary among target pathogens. In many organisms, SDHI resistance is attributed to single-point mutations in the *SdhB* gene leading to a substitution of a highly conserved histidine residue to tyrosine, arginine, leucine, or asparagine (2). This His residue, located in a region associated with a [3Fe-4S] high-potential non-heme iron sulfur-redox (S3) center, plays a key role not only in ubiquinone binding and reduction but also in carboxamide (such as carboxin and boscalid) proper binding and thus inhibitory action (13,14). Although Stammler (28) reported that mutations H272Y/R and P225L/F/T correlated with boscalid resistance in *B. cinerea*, no details were given about which mutation type was dominant and whether these mutation types occurred in field-resistant isolates. In the present study, we docu-

288-BS:	221	ST	S	P	S	Y	W	N	S	E	E	Y	L	G	P	A	I	L	L	Q	S	R	W	L	A	D	S	R	D	Q	K	E	E	R	K	A	A	L	D	N	S	M	S	L	Y	R	H	T	I	L	N	C	S	R	T	C	281
1753-BS:	221	ST	S	P	S	Y	W	N	S	E	E	Y	L	G	P	A	I	L	L	Q	S	R	W	L	A	D	S	R	D	Q	K	E	E	R	K	A	A	L	D	N	S	M	S	L	Y	R	H	T	I	L	N	C	S	R	T	C	281
1760-BS:	221	ST	S	P	S	Y	W	N	S	E	E	Y	L	G	P	A	I	L	L	Q	S	R	W	L	A	D	S	R	D	Q	K	E	E	R	K	A	A	L	D	N	S	M	S	L	Y	R	H	T	I	L	N	C	S	R	T	C	281
1924-BS:	221	ST	S	P	S	Y	W	N	S	E	E	Y	L	G	P	A	I	L	L	Q	S	R	W	L	A	D	S	R	D	Q	K	E	E	R	K	A	A	L	D	N	S	M	S	L	Y	R	H	T	I	L	N	C	S	R	T	C	281
6627-BS:	221	ST	S	P	S	Y	W	N	S	E	E	Y	L	G	P	A	I	L	L	Q	S	R	W	L	A	D	S	R	D	Q	K	E	E	R	K	A	A	L	D	N	S	M	S	L	Y	R	H	T	I	L	N	C	S	R	T	C	281
6628-BS:	221	ST	S	P	S	Y	W	N	S	E	E	Y	L	G	P	A	I	L	L	Q	S	R	W	L	A	D	S	R	D	Q	K	E	E	R	K	A	A	L	D	N	S	M	S	L	Y	R	H	T	I	L	N	C	S	R	T	C	281
6635-BS:	221	ST	S	P	S	Y	W	N	S	E	E	Y	L	G	P	A	I	L	L	Q	S	R	W	L	A	D	S	R	D	Q	K	E	E	R	K	A	A	L	D	N	S	M	S	L	Y	R	H	T	I	L	N	C	S	R	T	C	281
7435-BS:	221	ST	S	P	S	Y	W	N	S	E	E	Y	L	G	P	A	I	L	L	Q	S	R	W	L	A	D	S	R	D	Q	K	E	E	R	K	A	A	L	D	N	S	M	S	L	Y	R	H	T	I	L	N	C	S	R	T	C	281
7452-BS:	221	ST	S	P	S	Y	W	N	S	E	E	Y	L	G	P	A	I	L	L	Q	S	R	W	L	A	D	S	R	D	Q	K	E	E	R	K	A	A	L	D	N	S	M	S	L	Y	R	H	T	I	L	N	C	S	R	T	C	281
6632-BR:	221	ST	S	P	S	Y	W	N	S	E	E	Y	L	G	P	A	I	L	L	Q	S	R	W	L	A	D	S	R	D	Q	K	E	E	R	K	A	A	L	D	N	S	M	S	L	Y	R	R	T	I	L	N	C	S	R	T	C	281
7420-BR:	221	ST	S	P	S	Y	W	N	S	E	E	Y	L	G	P	A	I	L	L	Q	S	R	W	L	A	D	S	R	D	Q	K	E	E	R	K	A	A	L	D	N	S	M	S	L	Y	R	R	T	I	L	N	C	S	R	T	C	281
7434-BR:	221	ST	S	P	S	Y	W	N	S	E	E	Y	L	G	P	A	I	L	L	Q	S	R	W	L	A	D	S	R	D	Q	K	E	E	R	K	A	A	L	D	N	S	M	S	L	Y	R	R	T	I	L	N	C	S	R	T	C	281
7436-BR:	221	ST	S	P	S	Y	W	N	S	E	E	Y	L	G	P	A	I	L	L	Q	S	R	W	L	A	D	S	R	D	Q	K	E	E	R	K	A	A	L	D	N	S	M	S	L	Y	R	R	T	I	L	N	C	S	R	T	C	281
7392-BR:	221	ST	S	P	S	Y	W	N	S	E	E	Y	L	G	P	A	I	L	L	Q	S	R	W	L	A	D	S	R	D	Q	K	E	E	R	K	A	A	L	D	N	S	M	S	L	Y	R	R	T	I	L	N	C	S	R	T	C	281
7451-BR:	221	ST	S	P	S	Y	W	N	S	E	E	Y	L	G	P	A	I	L	L	Q	S	R	W	L	A	D	S	R	D	Q	K	E	E	R	K	A	A	L	D	N	S	M	S	L	Y	R	R	T	I	L	N	C	S	R	T	C	281
7453-BR:	221	ST	S	P	S	Y	W	N	S	E	E	Y	L	G	P	A	I	L	L	Q	S	R	W	L	A	D	S	R	D	Q	K	E	E	R	K	A	A	L	D	N	S	M	S	L	Y	R	R	T	I	L	N	C	S	R	T	C	281
7455-BR:	221	ST	S	P	S	Y	W	N	S	E	E	Y	L	G	P	A	I	L	L	Q	S	R	W	L	A	D	S	R	D	Q	K	E	E	R	K	A	A	L	D	N	S	M	S	L	Y	R	R	T	I	L	N	C	S	R	T	C	281
7494-BR:	221	ST	S	P	S	Y	W	N	S	E	E	Y	L	G	P	A	I	L	L	Q	S	R	W	L	A	D	S	R	D	Q	K	E	E	R	K	A	A	L	D	N	S	M	S	L	Y	R	R	T	I	L	N	C	S	R	T	C	281
7437-BR:	221	ST	S	P	S	Y	W	N	S	E	E	Y	L	G	P	A	I	L	L	Q	S	R	W	L	A	D	S	R	D	Q	K	E	E	R	K	A	A	L	D	N	S	M	S	L	Y	R	Y	T	I	L	N	C	S	R	T	C	281
7391-BR:	221	ST	S	P	S	Y	W	N	S	E	E	Y	L	G	P	A	I	L	L	Q	S	R	W	L	A	D	S	R	D	Q	K	E	E	R	K	A	A	L	D	N	S	M	S	L	Y	R	Y	T	I	L	N	C	S	R	T	C	281
7465-BR:	221	ST	S	P	S	Y	W	N	S	E	E	Y	L	G	P	A	I	L	L	Q	S	R	W	L	A	D	S	R	D	Q	K	E	E	R	K	A	A	L	D	N	S	M	S	L	Y	R	Y	T	I	L	N	C	S	R	T	C	281
7466-BR:	221	ST	S	P	S	Y	W	N	S	E	E	Y	L	G	P	A	I	L	L	Q	S	R	W	L	A	D	S	R	D	Q	K	E	E	R	K	A	A	L	D	N	S	M	S	L	Y	R	Y	T	I	L	N	C	S	R	T	C	281
8220-BR:	221	ST	S	P	S	Y	W	N	S	E	E	Y	L	G	P	A	I	L	L	Q	S	R	W	L	A	D	S	R	D	Q	K	E	E	R	K	A	A	L	D	N	S	M	S	L	Y	R	H	T	I	L	N	C	S	R	T	C	281

Fig. 1. Alignment of the deduced amino acid sequences (positions 221 to 281) of the *BcSdhB* gene from 14 boscalid-resistant (BR) and nine boscalid-sensitive (BS) isolates of *Botrytis cinerea*. The vertical box indicates the changes at amino acid positions 225 and 272 that were correlated with resistance to boscalid.

mented that the mutation H272R was the most frequent genotype in field Bos^R isolates of *B. cinerea* from apple and that mutation H272Y also was common in field Bos^R populations. More recently, H272Y has been reported to be the most frequent genotype in Bos^R populations of *B. cinerea* in French and German vineyards (22). It appears that the dominance of mutation types in *BcSdhB* is related to the populations in particular geographic regions and crop species.

Previous studies also indicated that proline at position 225 could be replaced by leucine, phenylalanine, or threonine in lab Bos^R mutants of *B. cinerea* (8,22,28). However, in our study, only one isolate carried the mutation P225F among the 66 field Bos^R isolates. This indicated that this mutation was not the dominant genotype in field-resistant isolates of *B. cinerea* from apple. The findings support the conclusion that each mutation found in one particular target-encoding gene can independently result in resistance to SDHIs (4,22,26). Single-point mutations in *SdhC* and *SdhD* have also been reported to be correlated with SDHI resistance in some fungal pathogens but these types of mutations are less common compared with those in *SdhB* (2,22). In our study, we did not sequence *BcSdhC* and *BcSdhD* because point mutations in these two genes are much less common and single-point mutations in *BcSdhB* were detected in all Bos^R isolates, which were consistent with previous reports (22,28). However, it remains to be determined whether frequency of different mutation types in *BcSdhB* will shift over time or whether mutants carrying different types of mutations in *BcSdhB*, *BcSdhC*, or *BcSdhD* will evolve if boscalid or other SDHIs continue to be used in the field.

In the present study, phenotyping of isolates for boscalid resistance and determination of EC₅₀ values of boscalid for resistant isolates were based on a conidial germination assay described previously (19). The results of molecular analysis of point muta-

Fig. 3. Frequency distribution of boscalid-resistant isolates of *Botrytis cinerea* exhibiting different levels of resistance expressed as resistance factor (folds of the concentration of boscalid that inhibits fungal growth by 50% relative to control [EC₅₀ value] for a resistant isolate relative to the mean EC₅₀ value of boscalid for the baseline sensitive isolates) between 19 type I mutants (carrying mutation H272Y in the *BcSdhB* gene) and 46 type II mutants (carrying mutation H272R in the *BcSdhB* gene).

Fig. 2. Specificity of **A**, the allele-specific polymerase chain reaction (PCR) primer pair 272Y/R-F + 272Y-R; **B**, the allele-specific PCR primer pair 272Y/R-F + 272R-R; and **C**, the multiplex allele-specific PCR primers 272Y/R-F + 272Y-R + 272R-R for detection of boscalid-resistant type I mutants (carrying mutation H272Y in *BcSdhB*) and type II mutants (carrying mutation H272R in *BcSdhB*) of *Botrytis cinerea*. The first lane ladder is the DNA marker and the last lane CK is a negative control without template DNA.

tions in *BcSdhB* were in agreement with those of phenotyping of boscalid resistance based on the conidial germination assay because point mutations in *BcSdhB* were observed in all Bos^R isolates but not Bos^S isolates. Our results supported the recommendation that the conidial germination assay on WA at the discriminatory concentration of 5 µg/ml is a reliable method for phenotyping *B. cinerea* isolates for resistance to boscalid (19).

Furthermore, we developed allele-specific PCR assays for detection of boscalid resistance in *B. cinerea* isolates carrying mutations at the codon position 272, which would facilitate fungicide resistance-monitoring efforts.

As expected, positive cross resistance had been demonstrated in some SDHIs, such as between flutolanil and carboxin in *C. cinereus* (16) and between boscalid and carboxin in *Alternaria*

Fig. 4. Electrophoretic separation of DNA fingerprints of *Botrytis cinerea* isolates amplified by the microsatellite primer (AAC)₅.

Fig. 5. Unweighted pairgroup method with arithmetic averages cluster analysis of microsatellite-primed polymerase chain reaction data for 95 *Botrytis cinerea* isolates, including 39 boscalid-resistant isolates (19 type I mutants carrying mutation H272Y in the *BcSdhB* gene, 19 type II mutants carrying mutation H272R in *BcSdhB*, and 1 type III mutant carrying mutation P225F in *BcSdhB*), and 56 boscalid-sensitive isolates from apple.

alternata (3). These reports suggested that boscalid and carboxin may share similar resistance mechanisms in fungal pathogens (3,16). Numerous studies have been done to elucidate molecular mechanisms of carboxin resistance (2,7,16,26,27). Cross resistance between boscalid and carboxin observed in *B. cinerea* in our study further supports our findings that point mutations in *BcSdhB* are correlated with boscalid resistance in field-resistant isolates of *B. cinerea* from apple. In recent years, more new SDHI fungicide have been discovered (10) and some of them, such as fluopyram and penthiopyrad, are currently in the process of registration for commercial use in the United States for control of gray mold and other diseases. In *A. alternata*, Bos^R isolates exhibited cross resistance to penthiopyrad but remained sensitive to fluopyram (1). In *B. cinerea*, negative cross resistance between boscalid and fluopyram has been reported (20). Different cross-resistance patterns between boscalid and other SDHIs suggest that different molecular mechanisms may be involved in resistance to different subclasses of SDHI fungicides. More research is still needed to understand molecular mechanisms of resistance to newly introduced SDHIs. Such information is important to management of SDHI resistance in *B. cinerea*.

Part of our objective was to determine whether different phenotypes of boscalid resistance (levels of resistance) are associated with particular types of point mutations in *BcSdhB* among Bos^R isolates. In the present study, Bos^R isolates were grouped into four resistant phenotypes based on their levels of resistance to boscalid. Our results indicated that, among Bos^R isolates, Bos^R phenotypes (levels of resistance) were not associated with particular types of point mutations (H272R versus H272Y) in *BcSdhB* because all four Bos^R phenotypes were present in isolates carrying either the H272R or H272Y mutation. In other words, mutations H272R and -Y resulted in phenotypes ranging from low resistance to very strong resistance. Previously, Stammler (28) reported that Bos^R isolates of *B. cinerea* carrying mutation H272Y exhibited more pronounced resistance than isolates containing mutation H272R. In contrast, De Miccolis Angelini et al. (8) reported that point mutation H272Y was correlated with low boscalid resistance in *B. cinerea* but their results were based only on lab UV-induced mutants, whereas Leroux et al. (22) reported that *B. cinerea* isolates carrying H272Y and H272R were all moderately resistant to boscalid but their observations were based on a limited number of isolates. The discrepancy between our results and those previously reported could be due to the differences between lab UV-induced mutants and field-resistant isolates, sample sizes, and populations of the pathogen from different crop species in different geographical regions. Different levels of boscalid resistance present among *B. cinerea* isolates carrying mutations at position 225 (P225L/F/T) have also been reported (28). In our study, only one isolate had the mutation at codon 225 (P225F). Thus, we were not able to assess the relationships between the mutations at position 225 and levels of boscalid resistance in field-resistant isolates.

Varying levels of boscalid resistance in isolates within the same mutation type in *BcSdhB* of *B. cinerea* may be due to the nature of great genetic variability among the isolates of the pathogen (5). In *A. alternata*, Avenot et al. (3) found no differences in level of boscalid resistance between Bos^R isolates carrying mutation H277Y and those carrying mutation H277R in the *AaSdhB* gene. However, unlike our study, no differences in level of boscalid resistance were observed in *A. alternata* within the same mutation type because all isolates were classified highly resistant to boscalid (3). Similarly, no differences in level of carboxin resistance were observed in the mutants of *Aspergillus oryzae* carrying mutations in the *SdhB* gene (26). However, mutants carrying mutations in *SdhC* and *SdhD* genes exhibited a lower level of resistance to carboxin than the mutants carrying mutations in the *SdhB* gene. It appeared that a single point mutation in *SdhB*

resulting in varying levels of boscalid resistance (low to strong) may be pathogen dependent.

We detected a high level of genetic variation in populations of *B. cinerea* from apple based on the MP-PCR analysis. Our results were in agreement with previous studies on genetic structure analysis of *B. cinerea* from other crops (24,25). In our study, *B. cinerea* mainly were divided into three groups based on the MP-PCR analysis. Bos^R isolates were clustered with some Bos^S isolates into either group A or group B, and no Bos^R isolates were clustered into group C. This may indicate that the likelihood for the wild-type isolates in groups A and B to develop resistance to boscalid is higher than those in group C. However, within Bos^R populations, no association was found between types of mutations in the *BcSdhB* gene and genetic groups based on the MP-PCR analysis because both type I and II mutants were present in either group A or group B. Our results may suggest that the development of resistance to boscalid in *B. cinerea* likely is not totally random, and resistant populations may come from different specific genetic groups.

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