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Characterization and Genetic Diversity of Benzimidazole-resistant and -sensitive *Monilinia fructicola* Isolates in Korea

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To characterize benzimidazole-resistant and -sensitive *Monilinia fructicola* populations, the fungal isolates were obtained from peach plants showing brown rot and bloosom blight. Benzimidazole-sensitive isolates did not grow on potato dextrose agar (PDA) amended with ≥1.0 μg active ingredient (a.i.)/ml of the fungicides. However, benzimidazole-resistant isolates grew on PDA regardless of the tested concentrations of fungicides. Benzimidazole-resistant isolates did not grow on diethofencarb-PDA, but sensitive isolates grew on the same PDA. In the nucleotide sequences of β-tubulin gene, only codon 198 (GAG: glutamic acid), a target site for benzimidazole, was replaced with GCG (alanine) in all of the resistant isolates, and this substitution seems to play an important role in the development of resistance. Other interesting codons such as 165 (GCT), 200 (TTC), and 241 (GCT) were not changed among the isolates. Benzimidazole-resistant and -sensitive isolates were clustered clearly in random amplified polymerphic DNA analyses and the results revealed that low levels of genetic diversity between benzimidazole-sensitive and -resistant isolates of *M. fructicola* in the investigated regions.

Keywords : benzimidazole-resistance, genetic diversity, *Monilinia fructicola*

Brown rot and blossom blight, caused by *Monilinia fructicola*, is one of destructive diseases of stone fruits including peach, plum, and apricot in Korea (Lim et al., 1998; 1999). The disease is managed by cultural practices and application of fungicidal spray during blossoming and the ripening period (Agrios, 1997; Ogawa et al., 1984; 1985). The advent of many effective fungicides including benzimidazole had resulted in a reasonable control of the diseases (Delp, 1988). Benzimidazole fungicides were introduced in 1970s to control various fungal diseases on plants. However, frequent use of those fungicides has lead to the development of resistance to fungicides among the fungal plant pathogens. In the past two decades, fungal resistance to the fungicides has created an increasingly serious problem (Delp, 1988; McGrath, 2001). Around the world, many plant pathogens such as *Botrytis cinerea*, *Penicillium digitatum*, *P. italicum*, *Venturia inaequalis*, *Uncinula nectater*, *Coccomyces hiemalis*, and *Didymella bryoniae* have been reported for their resistance against benzimidazole fungicides (Bus et al., 1991; Jones and Ehret, 1980; Keinath and Zitter, 1998; Kim et al., 1995; Koneraadt et al., 1992; Pearson and Taschenberg, 1980; Wild and Eckert, 1982). Benzimidazoles inhibit polymerization of tubulin by binding with β-tubulin in the fungicide-sensitive isolates. While the fungicide-resistant isolates exhibited a reduced binding affinity of fungicide with β-tubulin (Yarden and Katan, 1993). A reduced binding affinity has been known to be associated with point mutation in the β-tubulin gene (Yarden and Katan, 1993). In *M. fructicola*, benzimidazole-resistance has been occasionally reported so far since it was first reported in 1970s (Elmer and Gaunt, 1994; Jones and Ehret, 1976; Ogawa et al., 1984; Sanamuang and Gaunt, 1995; Sonada and Ogawa, 1982; Tate et al., 1974). In Korea, there had been several reports on benzimidazole-resistances of fungi (Lim et al., 1998; 1999). Lack of information on the molecular properties of benzimidazole-resistance led us to carry out the present study.

To control benzimidazole-resistant isolates, diethofencarb, which is showing negatively correlated cross-resistance to benzimidazoles, is used as mixture but the incidence of isolates that are resistant to both benzimidazole and diethofencarb is a new problem in plant diseases control (Kim et al., 1995).

The objective of this study is to investigate genetic diversity between benzimidazole-resistant and -sensitive *M. fructicola* isolates from peach by identifying the β-tublin gene mutation site and determining sensitivity of the benzimidazole-resistant isolates to diethofencarb as well as attempting random amplified polymorphic DNA (RAPD) analysis.

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Materials and Methods

Isolation of *M. fructicola*. Fruits, stems, flowers, and mummified fruits with brown rot and blossom blight were collected from peach trees in commercial orchards at different locations in Chochiwon, Chongdo, Gyeongsan, and Youngduk. Those orchards had applied fungicides for several years to control several diseases. To obtain monoconidial isolate, agar blocks containing a germinating conidium were cut with a steel needle and transferred on potato dextrose agar (PDA). After incubation for 5 days at 25°C, monoconidia cultures were used in further experiments.

Sensitivity to fungicides. The following fungicides were used; benomyl (50% WP; wettable powder), carbendazim (60% WP), thiophanate-methyl (70% WP), and diethofencarb (technical grade) (Table 1). Fungicide suspensions prepared in sterilized distilled water and acetone were diluted to the concentrations with sterilized distilled water using micro-pipette, and added to PDA immediately before pouring into Petri plates (Tate et al., 1974). Sensitivity was tested with growth on fungicide-free PDA (check) and on PDA amended with 0.1, 1.0, 10, 100, and 1,000 mg active ingredient (a.i.)/ml of each fungicide. The 5-mm-diameter mycelial plugs were taken from the margin of 7-day-old PDA culture and transferred to the test media using a transfer-needle. The colony diameters were examined after incubation for 7 days at 25°C. The resistance to benzimidazoles was determined at 1.0 mg a.i./ml based on the results of previous studies (Lim et al., 1998; Ogawa et al., 1984).

DNA extraction, amplification and sequence of β-tubulin gene. Fungal DNA was extracted as previously described (Pollatro and Faretra, 1992). PCR amplification of β-tubulin gene was conducted in a final volume of 50 μl containing 0.4 pM oligonucleotide primer (GEN C; 5'-GAG GAA TTC CCA GAC CGT ATG ATG-3' and GEN D; 5'-GCT GGA TCC TAT TCT TTG GGT CGA ACA T-3'), 200 μM each of the four dNTPs (deoxynucleoside triphosphates), 1.5 mM $MgCl_2$, 1 unit of ampli *Taq* DNA polymerase (Takara, Japan), 5 μl of Ampli *Taq* buffer, and 10 μg/μl of DNA template. Amplification was carried with a Perkin-Elmer GeneAmp PCR system 2400 as follows; 1 cycle of 94°C for 5 min and 45 cycles of 94°C for 1 min, 52°C for 1 min, 72°C for 2 min, and 72°C for 5 min. Expected PCR product was gel-purified with the DNA purification system (Promega Cat. #A7170) and cloned into the P^{GEM}-T easy vector and nucleotide sequence of the cloned PCR product were determined using ABI PRIAM DNA Analyzer (PE Biosystems) at Macrogen (Seoul, Korea). The deduced amino acid sequences of the cloned β-tubulin genes were compared between the benzimidazole-resistant and -sensitive isolates of *M. fructicola*.

RAPD analysis. RAPD was conducted in a final volume of 50 μl containing 1 μM oligonucleotide 10-base pair primers (Bioneer, Korea; Table 2), 200 μM each of the four dNTP, 1.5 mM $MgCl_2$, 0.5 unit of ampli *Taq* DNA polymerase (Takara, Japan), 5 μl of ampli *Taq* buffer, and 3.3 μg/μl of DNA template. Amplification was carried out using Perkin-

Table 1. Fungicides used in this study

Common name	Active Ingredient	Introduced Year	Recommended Use (Times)
Benomyl	Methyl-1(-butycarbomoxmyl)-2-benzimidazolecarbamate	1975	4-6
Carbendazim	Methyl benzimidazole-2-yl carbamate	1981	4-6
Thiophanate-methyl	1,2-bis (3-ethoxycarbonyl-2-thioueido) benzen	1973	4-6
Diethofencarb	Isopropyl-3,4-diethoxylphenyl carbamate	1992	6

Table 2. List of the 10 primers and their sequences used for RAPD analyses

Primer No.	Sequence (5' to 3')	G/C Contents (%)	No. of amplified bands	No. of polymorphic bands
N-0001	GAC GAA ACA T	40	6	1
N-8006	AGC CAG CGA A	60	11	5
N-8009	GGG TAA CGC C	70	10	7
N-8017	TGC TCT GCC C	70	12	10
N-8041	ATC GGG TCC G	70	9	5
N-8065	ACC GCC TGC T	70	7	6
N-8082	AAG GAT CAG A	40	6	3
Total	–	–	62	37

Elmer GeneAmp PCR system 2400 as follows; 1 cycle of 94°C for 5 min and 40 cycles of 94°C for 1 min, 37°C for 1 min, 72°C for 2 min. After 40 cycles, a final extension was conducted at 72°C for 10 min.

Results and Discussion

Sensitivity to fungicides. Benzimidazole-sensitive isolates did not grow on PDA amended with ≥1.0 μg a.i./ml of each fungicide. However, benzimidazole-resistant isolates grew on PDA amended with all tested concentrations (from 0.1 to 1000 μg a.i./ml) of the fungicides (Fig. 1) even though their growth was reduced to less than a half of the normal growth at the highest concentration (1000 μg a.i./ml). As Table 3 shows, all of the benzimidazole-resistant isolates examined were sensitive to diethofencarb, and all of the benzimidazole-sensitive isolates were resistant to diethofencarb. The results suggest that the use of diethofencarb may be efficient to control brown rot on stone fruits caused by benzimidazole-resistant *M. fructicola* if it does not have any phytotoxicity. However, the isolates resistant to two different fungicides were detected in field population of *Botrytis cinerea* causing gray mold on vegetable crop in Korea (Kim et al., 1995). Therefore, in the cases that diethofencarb is applied, it will be necessary to monitor sensitivity shift of *M. fructicola* periodically in field population. The use of reasonable fungicide application program is also required to maintain good control effect (Köller and Wilcox, 2001).

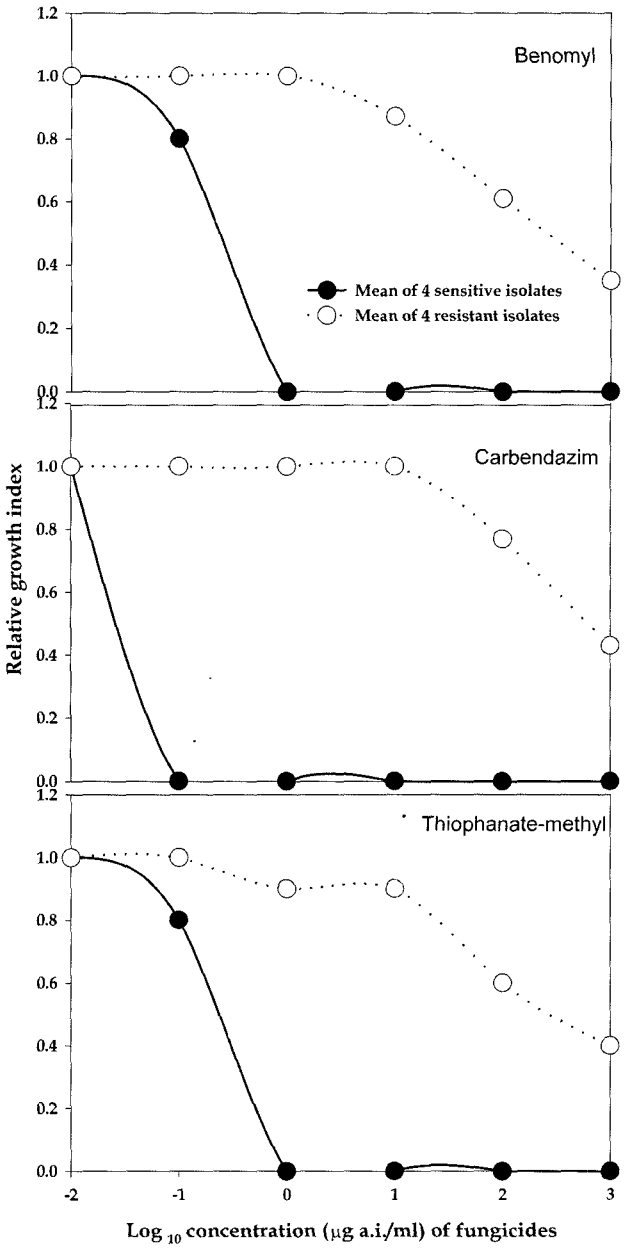

Fig. 1. Growth of benzimidazoles-resistant (○) and -sensitive (●) isolates of *Monilinia fructicola* on PDA amended with 0.01, 0.1, 1.0, 10, 100, and 1,000 μg a.i./ml of each fungicide.

Amplification and sequencing of β-tubulin gene. PCR with primers GEN C and GEN D yielded a 440-bp product from all tested *M. fructicola* isolates (Fig. 2). The PCR products were cloned into a plasmid vector and their

Table 3. Fungal isolates used in this study

Isolates	Regions	Responses to fungicides[a]	
		Carbendazim (1 μg a.i./ml)	Diethofencarb (1 μg a.i./ml)
CD0113	Chongdo	Sensitive	Resistant
CD0121	Chongdo	Sensitive	Resistant
CH0101	Chochiwon	Sensitive	Resistant
CH0105	Chochiwon	Sensitive	Resistant
CH0109	Chochiwon	Sensitive	Resistant
GY0110	Gyeongsan	Sensitive	Resistant
CH0111	Chochiwon	Sensitive	Resistant
CH0118	Chochiwon	Sensitive	Resistant
CH0121	Chochiwon	Sensitive	Resistant
CH0125	Chochiwon	Sensitive	Resistant
CD0102	Chongdo	Resistant	Sensitive
CD0106	Chongdo	Resistant	Sensitive
CD0118	Chongdo	Resistant	Sensitive
GY0102	Gyeongsan	Resistant	Sensitive
GY0103	Gyeongsan	Resistant	Sensitive
GY0104	Gyeongsan	Resistant	Sensitive
GY0107	Gyeongsan	Resistant	Sensitive
GY0101	Gyeongsan	Resistant	Sensitive
GY0112	Gyeongsan	Resistant	Sensitive
GY0115	Gyeongsan	Resistant	Sensitive

[a]The responses to fungicides were tested on PDA with and without fungicides.

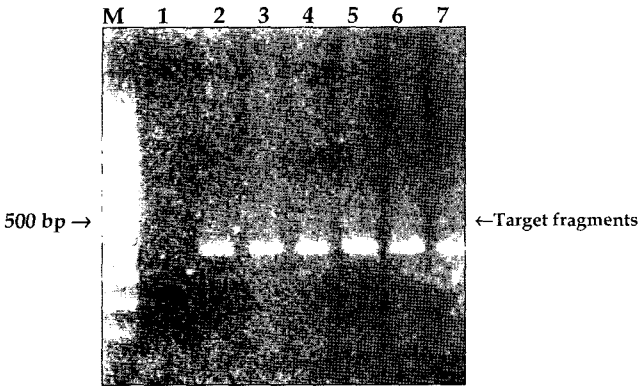

Fig. 2. Amplification of a part of β-tubulin gene with primer GEN C and GEN D from *M. fructicola* isolates. The PCR products were separated on a 1.5% agarose gel stained with ethidium bromide. Lanes M: molecular size marker (100 bp ladder), 1: Negative control, 2: CH0101, 3: GY0110, 4: GY0115, 5: CD0102, 6: GY0102, and 7: CD0118.

nucleotide sequences were determined. These analyses confirmed that the inserts were a 440-bp β-tubulin gene fragment, and exhibited high homology to the corresponding gene from *Botrytis cinerea* and *Venturia inaequalis* from (Yarden and Katan, 1993). Resistant isolates (GY0115, CD0102, GY0102, CD0118) showed replacement of a codon 198 from GAG (glutamic acid) with GCG (alanine) (Fig. 3). Other interesting codons such as 165 (GCT), 200 (TTC), and 241 (GCT) were not different between the resistant isolates and the sensitive isolates (data not shown). These results were not consistent with the earlier reports (Koenraadt et al., 1992; Yarden and Katan, 1993). In general, benzimidazole resistant-isolates carried a substitution with lysine or alanine at codon 198 or tyrosine at codon 200, while the isolates resistant to both benzimidazole and diethofencarb carried a substitution with tyrosine at codon 200 (Yarden and Katan, 1993). These

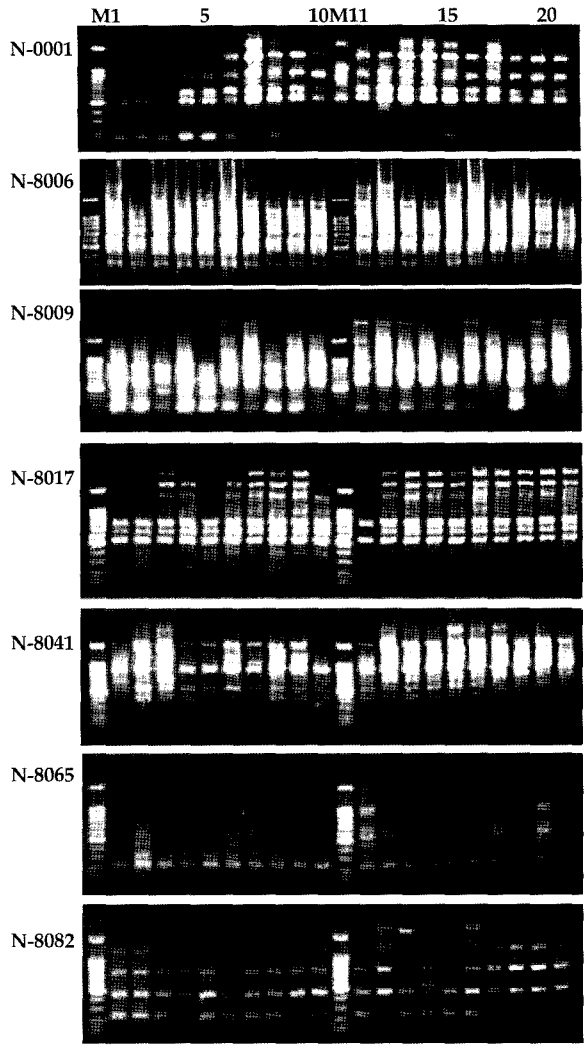

Fig. 4. PCR amplified DNA fragments using the primers (left) listed in Table 2. The numbers above the lane indicate the *M. fructicola* isolates shown in Table 3. Lane M: molecular size marker, lanes 1 to 10: sensitive isolates, and lanes 11 to 20: resistant isolates.

Positions	187	188	189	190	191	192	193	194	195	196	197	198	199	200	201
B.cinerea	L	S	V	H	Q	L	V	E	N	S	D	E	T	F	C
V.inaequalis	L	S	V	H	Q	L	V	E	N	S	D	E	T	F	C
N.crassa	L	S	V	H	Q	L	V	E	N	S	D	E	T	F	C
B.cinerea (Ben^S)	L	S	V	H	Q	L	V	E	N	S	D	E	T	F	C
B.cinerea (Ben^R)	L	S	V	H	Q	L	V	E	N	S	D	**A/K**	T	**Y**	C
CH0101 (Ben^S)	L	S	V	H	Q	L	V	E	N	S	D	E	T	F	C
GY0110 (Ben^S)	L	S	V	H	Q	L	V	E	N	S	D	E	T	F	C
GY0115 (Ben^R)	L	S	V	H	Q	L	V	E	N	S	D	**A**	T	F	C
CD0102 (Ben^R)	L	S	V	H	Q	L	V	E	N	S	D	**A**	T	F	C
GY0102 (Ben^R)	L	S	V	H	Q	L	V	E	N	S	D	**A**	T	F	C
CD0118 (Ben^R)	L	S	V	H	Q	L	V	E	N	S	D	**A**	T	F	C

Fig. 3. Comparison of deducted amino acid sequences of the β-tubulin gene from several fungi (Yarden and Katan, 1993) including *Monilinia fructicola* isolates. Ben^S and Ben^R in the parentheses represent sensitivity and resistance to benzimidazole respectively. Bold letters represent changed amino acids.

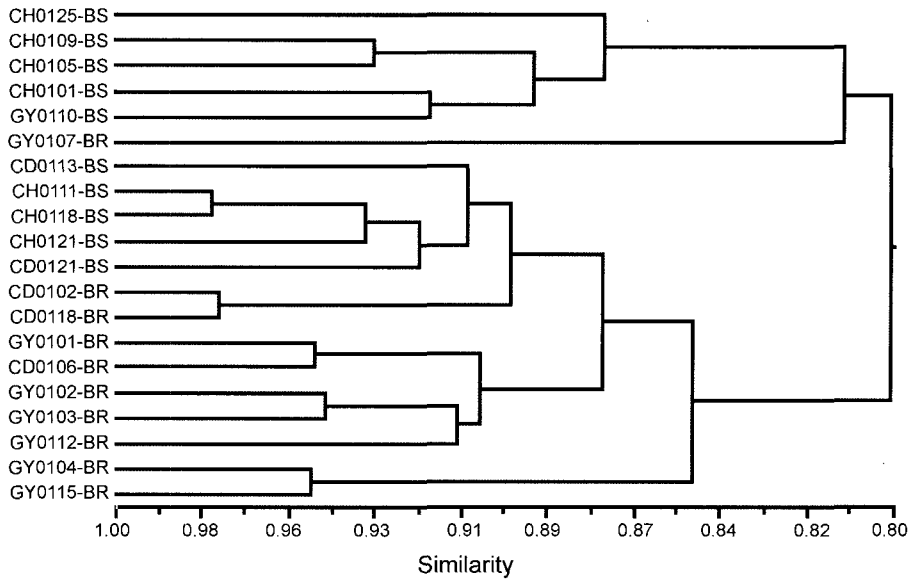

Fig. 5. UPGMA dendrogram showing the relationship among the 20 isolates of *M. fructicola* based on the polymorphic bands shown on an 1.5% agarose gel in RAPD analyses. BS: benzimidazole-sensitive, BR: benzimidazole-resistant.

results imply that the substitution at codon 198 in *M. fructicola* must play an important role in the development of benzimidazole-resistance in Korea.

RAPD analysis. To investigate the genetic diversity of benzimidazole-resistant and-sensitive isolates, RAPD was performed using 10 arbitrary primers. Among the primers, seven of them generated reproducible banding patterns and they were selected for RAPD analyses (Table 2). A total of 62 distinct bands were amplified using the DNAs from 20 isolates and thirty-seven of them showed polymorphism (Table 2). The sizes of the RAPDs ranged from 150 bp to more than 1,500 bp (Fig. 4). At 80% homology level, all isolates belonged to one cluster and benzimidazole-resistant and –sensitive isolates were recognized well in the cluster. CD0102 and CD0118, benzimidazole-resistant isolates from Chongdo, belonged to the same cluster with all the remaining sensitive isolates at 90% similarity (Fig. 5). All the remaining resistant isolates showed the similarity over 90% and the isolates belonged to the same cluster at 85% with the other resistant isolates CD0102 and CD0118 (Fig. 5). However, exceptionally, benzimidazole-resistant isolate GY0107 from Gyeongsan belonged to the same cluster with benzimidazole-sensitive isolates such as CH0125, CH0109, CH0105, CH0101, and GY0110 of Chochiwon and Gyeongsan at 81% homology level (Fig. 5). The results indicate that genetic diversity is generally low among benzimidazole-sensitive and -resistant isolates of *M. fructicola* examined, consistent with the reports of Lee et al. (1998) and Li et al. (1999). The difference in detection rates of benzimidazole-resistance with locations may be attributed to the history of fungicides application in the field, disease pressure, and environmental conditions.

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