Occurrence and Detection of the DMI Resistance-Associated Genetic Element 'Mona' in *Monilinia fructicola*

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ABSTRACT

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Sterol demethylation inhibitor (DMI) fungicide resistance in isolates of *Monilinia fructicola* from Georgia has been linked to overexpression of the *MfCYP51* gene and a corresponding 65-bp genetic element 'Mona' inserted in the upstream region of *MfCYP51*. In this study, a polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method was developed to detect the Mona element. Fourteen DMI fungicide-resistant (DMI-R) and six DMI fungicide-sensitive (DMI-S) isolates from Georgia, six DMI-R and 11 DMI-S isolates from South Carolina, seven DMI-R and nine DMI-S isolates from New York, and two DMI-R and three DMI-S isolates from Ohio were used in this study. The isolates from the southeastern United States and Ohio were collected from peach, whereas isolates from New York were collected from cherry. A 376-bp fragment containing the Mona element was consistently amplified with primer pair *INS65-F* and *INS65-R* from DMI-R isolates, and either a 311-bp or 1,815-bp fragment was amplified from DMI-S isolates. The primer pair did not amplify DNA fragments of similar sizes from isolates of five other common fruit rot pathogens of peach, including *Alternaria alternata*, *Colletotrichum acutatum*, *Gilbertella persicaria*, *Penicillium expansum*, and *Rhizopus stolonifer*. Gel electrophoresis of the PCR amplicon can distinguish between DMI-R and DMI-S isolates based on the 65-bp size difference of the amplicon; however, the restriction digestion assay can verify questionable results, especially in the absence of a positive control. Only the 376-bp fragment containing the Mona element was digestible with endonuclease *BsrBI*, resulting in two restriction fragments of 236 and 140 bp in size. In this study, a protocol for Mona detection from aerial fungal structures was developed that can yield results within a few hours of sampling. This study confirms that the Mona element is strongly linked to the DMI-resistance phenotype and reveals that overexpression of the *MfCYP51* gene is a common DMI fungicide resistance mechanism in *M. fructicola*, not only in Georgia but throughout the eastern United States.

Additional keywords: brown rot, molecular markers, propiconazole, stone fruits

Sterol demethylation inhibitor (DMI) fungicides have been widely used for more than 20 years to control brown rot of stone fruits caused by *Monilinia* spp. As a result, resistance to DMI fungicides was detected in *M. fructicola* isolates from commercial peach orchards in Georgia (12), and more recently from commercial cherry orchards in New York (10). DMI fungicide resistance was suspected in *M. fructicola* from commercial peach orchards in Ohio (K. Cox & M. Ellis, *personal communication*).

Products from other chemical classes, such as benzimidazoles, anilinopyrimidines, quinone outside inhibitors (respiration inhibitors), and anilides, are also available for brown rot control in commercial stone fruit orchards of the United States. However, resistance concerns (12,15), registration limitations, and/or lack of efficacy (6,7,12) limit the use of the aforementioned chemical classes for control. Consequently, the DMI fungicides will remain an important tool for brown rot management in stone fruits in the foreseeable future.

Several mechanisms of resistance to DMI fungicides have been described in plant pathogenic fungi. The most common of those mechanisms found in field isolates of plant pathogens include mutations in and overexpression of the cytochrome P450 14 α -demethylase (*CYP51*) gene encoding the target enzyme of azole fungicides (5,14). In a recent study, overexpression of the *CYP51* gene in *M. fructicola* isolates from Georgia correlated with propiconazole resistance (8). This overexpres-

sion was associated with a repetitive genetic element called 'Mona' located upstream of the *MfCYP51* gene. The Mona element contains a predicted promoter sequence, which likely triggers the elevated expression of *MfCYP51* (8).

The goal of this study was to develop a molecular assay to quickly identify the Mona element located upstream from *MfCYP51* in *M. fructicola* and to screen a larger collection of isolates from the eastern United States with varying DMI resistance phenotypes.

MATERIALS AND METHODS

Isolates. A total of 58 single-spore *M. fructicola* isolates resistant (DMI-R) or sensitive (DMI-S) to propiconazole were used in this study (Table 1). These isolates were collected from 10 locations in 10 counties of four states including Georgia, South Carolina, New York, and Ohio. Whenever possible, DMI-R and DMI-S isolates were included from the same location to ensure similar genetic backgrounds. Isolates from South Carolina, Georgia, and Ohio were collected from peach fruit, whereas isolates from New York were collected from cherry fruit as described previously (12). All isolates were kept on potato dextrose agar (PDA; EMD Chemicals, Gibbstown, NJ) slants at 4°C under sterile mineral oil. In addition to *M. fructicola*, five other common fruit pathogens (*Alternaria alternata*, *Colletotrichum acutatum*, *Gilbertella persicaria*, *Penicillium expansum*, and *Rhizopus stolonifer*) were included. These isolates were obtained from either commercial or experimental orchards in South Carolina and Georgia.

Sensitivity of *M. fructicola* isolates to propiconazole. The sensitivity of the *M. fructicola* isolates to the DMI fungicide propiconazole (PropiMax EC; Dow Agro-Sciences LLC, Indianapolis, IN) was determined, as previously described (1). The relative mycelial growth was expressed as the growth on 0.3 μ g/ml propiconazole-amended PDA as a percentage of the growth on nonamended PDA. The discriminatory dose of 0.3 μ g/ml was shown previously to distinguish between baseline (sensitive) and resistant populations of *M. fructicola* (1).

Extraction of genomic DNA. Each fungal isolate was grown in 40 ml of potato

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dextrose broth (PDB; Difco Laboratories, Sparks, MD) for 5 days at 22°C on an orbital shaker (120 rpm). Mycelium was harvested, frozen in liquid nitrogen, and ground into fine powder with mortar and pestle. Genomic DNA was isolated from the powdered mycelium using the DNeasy

Plant Mini Kit (Qiagen Inc., Valencia, CA) according to the manufacturer's manual.

Development of a PCR-based method to detect the *Mona* element located upstream of *MfCYP51* and validation of primer specificity. PCR primers *INS65-F* (5'-TCG ATG TTG CGT GGT CTG TCA-

3') and *INS65-R* (5'-CGG AGA GCG TCG ATG TAG GTG-3') were designed using the software DNASTAR (DNASTAR Inc., Nevada City, CA) based on a previously cloned *MfCYP51* upstream sequence (GenBank accession number EU035301). PCR reaction volumes were 25 µl contain-

Table 1. Characteristics of *Monilinia fructicola* isolates from Georgia, South Carolina, New York, and Ohio, and results of polymerase chain reaction (PCR) amplification and restriction digestion of a portion of the *MfCYP51* gene upstream sequence

Isolate	Origin	Host	Year of isolation	Sensitivity phenotype ^a	PCR fragment size (bp) ^b	<i>Bsr</i> BI restriction site ^c
Bmpc7	Byron, GA	Peach	2006	R (29.7)	376	+
Bmpc10	Byron, GA	Peach	2006	R (30.8)	376	+
Bmpc13	Byron, GA	Peach	2006	R (29.5)	376	+
BGA7	Byron, GA	Peach	2004	R (49.7)	376	+
BGA17	Byron, GA	Peach	2004	R (45.0)	376	+
BGA25	Byron, GA	Peach	2004	S (2.6)	311	—
GADL7	Fort Valley, GA	Peach	2003	R (38.7)	376	+
GADL129	Fort Valley, GA	Peach	2003	R (55.6)	376	+
GADL133	Fort Valley, GA	Peach	2003	R (58.8)	376	+
GADL193	Fort Valley, GA	Peach	2003	R (35.0)	376	+
GADL3	Fort Valley, GA	Peach	2003	S (0.0)	311	—
GADL161	Fort Valley, GA	Peach	2003	S (3.7)	311	—
GAAP3	Macon, GA	Peach	2003	R (47.9)	376	+
GAAP4	Macon, GA	Peach	2003	R (28.7)	376	+
GAAP5	Macon, GA	Peach	2003	R (36.3)	376	+
GAAP6	Macon, GA	Peach	2003	R (21.8)	376	+
GAAP10	Macon, GA	Peach	2003	R (37.4)	376	+
GAAP11	Macon, GA	Peach	2003	S (0.0)	311	—
GAAP12	Macon, GA	Peach	2003	S (0.0)	311	—
GAAP33	Macon, GA	Peach	2003	S (13.7)	311	—
SCEG5	Edgefield, SC	Peach	2007	R (70.6)	376	+
SCEG6	Edgefield, SC	Peach	2007	R (68.6)	376	+
SCEG9	Edgefield, SC	Peach	2007	R (46.1)	376	+
SCEG11	Edgefield, SC	Peach	2007	R (70.6)	376	+
SCEG12	Edgefield, SC	Peach	2007	R (74.5)	376	+
SCEG13	Edgefield, SC	Peach	2007	R (47.1)	376	+
SC99A3	Edgefield, SC	Peach	2004	S (5.0)	311	—
SC99A4	Edgefield, SC	Peach	2004	S (1.3)	311	—
SCBS1	York, SC	Peach	2003	S (0.9)	311	—
SCBS2	York, SC	Peach	2003	S (7.1)	311	—
SCBS7	York, SC	Peach	2003	S (0.7)	311	—
SCBS14	York, SC	Peach	2003	S (17.6)	311	—
SCCC8	Saluda, SC	Peach	2003	S (0.0)	311	—
SCCC12	Saluda, SC	Peach	2003	S (0.0)	311	—
SCDL21	Anderson, SC	Peach	2001	S (5.1)	311	—
SCDL28	Anderson, SC	Peach	2001	S (0.6)	311	—
SCDL72	Anderson, SC	Peach	2001	S (3.1)	311	—
NY1A	Geneva, NY	Cherry	2007	R (48.1)	376	+
NY1C	Geneva, NY	Cherry	2007	R (40.6)	376	+
NY2F	Geneva, NY	Cherry	2007	R (31.9)	376	+
NY4B	Geneva, NY	Cherry	2007	R (55.5)	376	+
NY4C	Geneva, NY	Cherry	2007	R (50.7)	376	+
NY6B	Geneva, NY	Cherry	2007	R (46.2)	376	+
NY6C	Geneva, NY	Cherry	2007	R (34.7)	376	+
NY3B	Geneva, NY	Cherry	2007	S (1.6)	1,815	—
NY3C	Geneva, NY	Cherry	2007	S (6.8)	1,815	—
NY7B	Geneva, NY	Cherry	2007	S (7.2)	1,815	—
NY7C	Geneva, NY	Cherry	2007	S (3.9)	1,815	—
NY8C	Geneva, NY	Cherry	2007	S (9.2)	311	—
NY9C	Geneva, NY	Cherry	2007	S (3.5)	1,815	—
NY10B	Geneva, NY	Cherry	2007	S (0.7)	1,815	—
NY12B	Geneva, NY	Cherry	2007	S (8.9)	1,815	—
NY13B	Geneva, NY	Cherry	2007	S (2.6)	1,815	—
OH1P	Berlin Heights, OH	Peach	2006	R (41.8)	376	+
OH3P	Berlin Heights, OH	Peach	2006	S (3.8)	311	—
OH8P	Rittman, OH	Peach	2006	R (37.8)	376	+
OH6P	Rittman, OH	Peach	2006	S (0.6)	311	—
OH10P	Rittman, OH	Peach	2006	S (9.6)	311	—

^a An isolate was considered resistant (R) when the mycelial growth was equal to or greater than 20% at the discriminatory dose of 0.3 µg/ml propiconazole relative to growth on nonamended medium; all other isolates were considered sensitive (S). Relative growth at 0.3 µg/ml propiconazole is shown in parentheses.

^b PCR fragment was amplified from genomic DNA using primers *INS65-F* and *INS65-R*.

^c “+” or “—” indicates presence or absence of the *Bsr*BI restriction site within the 376-bp PCR fragment, respectively.

ing 1× PCR buffer provided by the manufacturer, 20 ng genomic DNA, 0.5 μM of each primer, 200 μM of each dNTP, and 0.625 unit of *Taq* DNA polymerase (New England BioLabs, Ipswich, MA). Amplifications were performed in an “iCycler” thermal cycler (Bio-Rad Laboratories Inc., Hercules, CA) programmed for 3 min at 94°C, 30 cycles of 1 min at 94°C, 1 min at 60°C, 1 min at 72°C, followed by a final extension step of 5 min at 72°C. The PCR products were separated on a 2.0% agarose gel in 0.5× TBE buffer (44.5 mM Tris-borate, 1 mM EDTA, pH 8.0) at 100 V for 1 h. Gels were photographed with an Electrophoresis Documentation and Analysis System (KODAK EDAS 290, Eastman Kodak Company, New Haven, CT).

Amplification of the expected DNA fragments was confirmed by sequencing the fragments from seven DMI-R and three DMI-S isolates from Georgia, three DMI-R and three DMI-S isolates from South Carolina, one DMI-R and four DMI-S isolates from New York, and two DMI-R and two DMI-S isolates from Ohio. PCR products were purified using the QiaQuick Gel Extraction Kit (Qiagen Inc., Valencia, CA) and subsequently sequenced with BigDye Terminator v3.1 Kit (Applied Biosystems Inc., Foster City, CA) using primers *INS65-F* and *INS65-R*. All sequencing reactions were performed at the Clemson University Genomics Institute (CUGI).

The specificity of this primer pair to *M. fructicola* DNA was also examined on two isolates from each of five fungal species causing peach rots: *A. alternata*, *C. acutatum*, *G. persicaria*, *P. expansum*, and *R. stolonifer*. PCR amplification and gel electrophoresis analysis was conducted as described above.

Restriction fragment length polymorphism (RFLP) analysis of the PCR fragment amplified with primers *INS65-F* and *INS65-R*. A single *BsrBI* (New England BioLabs) restriction site was identified within the Mona element using the software DNAssist version 2.0 (DNAssist cc, Bellville, South Africa). PCR fragments were purified with the QiaQuick Gel Extraction Kit (Qiagen), and restriction digestions were performed at 37°C for 16 h according to the manufacturer’s recommendations (New England BioLabs). Both undigested and digested PCR products were separated on a 2.0% agarose gel, and the gel was photographed as described above.

To investigate whether the RFLP analysis could be simplified, the unpurified PCR fragment from DMI-R isolate GADL7 was digested with shortened incubation times. Digestions were conducted at 37°C for 0, 0.5, 1, 2, 4, and 16 h in 20-μl digestion volume including 2 μl of 10× NEBuffer 2 provided by the manufacturer, 0.5 μl of *BsrBI* enzyme (5 unit/reaction), and 17.5 μl of fresh PCR product. After the designated digestion

time, *BsrBI* was inactivated by adding 0.5 μl of 0.5 M EDTA, followed by heat inactivation at 80°C for 20 min. The digested PCR fragments were separated on a 2.0% agarose gel and photographed as described above.

Potential adaptation of this RFLP analysis to field samples was investigated using aerial fungal structures from isolates growing on agar medium and peach fruit. Aerial fungal structures (1 to 5 mg; mainly mycelium and conidia) from 39 NY cherry *M. fructicola* isolates (NY1-13A-C) growing on PDA or artificially inoculated cherries (isolates NY1A, -1C, -8B, and -8C) and four SC peach isolates (SCEG.5, -6, -9, and SC99A3) growing on artificially inoculated peach fruit (12) were removed without touching either the PDA or the peach fruit tissue using sterile toothpicks. Structures were placed into sterile 0.2 ml PCR tubes containing 5 μl of the Lyse-N-GO PCR Reagent (Pierce, Rockford, IL). Samples were then processed according to the manufacturer’s instructions, and subsequent PCR-RFLP analysis for detection of Mona was performed as described above. The digested PCR fragments were separated on a 2.0% agarose gel for 1 h at 100 V and photographed.

RESULTS

Sensitivity of *M. fructicola* isolates to propiconazole. Isolates resistant to the DMI fungicide propiconazole were collected from three locations in Georgia (Byron, Fort Valley, and Macon), one location in South Carolina (Edgefield), one location in New York (Geneva), and two locations in Ohio (Berlin Heights and Rittman). These locations were chosen be-

cause brown rot developed on fruit despite proper preharvest application of DMI fungicides. Relative growth values for resistant isolates at the discriminatory dose of 0.3 μg/ml propiconazole ranged from 21.8 to 74.5% (Table 1). DMI-S isolates were included from each of these locations and from three additional locations in South Carolina where DMI-R isolates have not yet been identified. Relative growth of the DMI-S isolates at the discriminatory dose of 0.3 μg/ml propiconazole ranged from 0 to 17.6% (Table 1).

Development of a PCR-based method to detect the Mona element located upstream of *MfCYP51* and validation of primer specificity. The primer pair *INS65-F* and *INS65-R* was designed based on the flanking sequence (GenBank accession number EU035301) of the Mona element located upstream of *MfCYP51*. The primers amplified a 376-bp fragment from all DMI-R isolates regardless of their origin and a 311-bp fragment from most DMI-S isolates (Table 1; Fig. 1). Except for the isolate NY8C, the primer pair amplified a single 1,815-bp fragment from all DMI-S cherry isolates from New York (Table 1; Fig. 1). Sequencing the 1,815-bp fragment from three representative isolates revealed that it contained an insert 1,504 bp in length. However, no portion of the sequence was homologous to the Mona element (data not shown). The primer pair did not amplify DNA of the relevant sizes from two isolates of each of five other common stone fruit pathogens. We did notice, however, weak PCR amplification of an approximate 1,500-bp product from the DNA of two *P. expansum* isolates (Fig. 2). This fragment, however, was not

Fig. 1. Gel electrophoresis analysis of undigested and *BsrBI* digested polymerase chain reaction (PCR) fragments amplified from *Monilinia fructicola* DMI-R and DMI-S isolates with primers *INS65-F* and *INS65-R*. Lanes 1 and 5, *EZ load* 100-bp molecular ruler (Bio-Rad Laboratories) and *Quick Load 1kb DNA Ladder* (New England BioLabs), respectively; lanes 2 to 4, undigested PCR fragments from DMI-R isolate NY2F, and DMI-S isolates OH3P and NY3C; lanes 6 to 8, *BsrBI* digested PCR fragments from DMI-R NY2F, and DMI-S isolates OH3P and NY3C.

Fig. 2. Gel electrophoresis analysis of polymerase chain reaction (PCR) fragments from six fungi using primers *INS65-F* and *INS65-R*. M = *exACTGene* Low Range DNA Ladder (Fisher Scientific); lanes 1 and 2, *Alternaria alternata* isolates 1 and 2; lanes 3 and 4, *Colletotrichum acutatum* isolates 1 and 2; lanes 5 and 6, *Gilbertella persicaria* isolates 1 and 2; lanes 7 and 8, *Penicillium expansum* isolates 1 and 2; lanes 9 and 10, *Rhizopus stolonifer*, isolates 1 and 2; and lanes 11 and 12, *Monilinia fructicola* isolates GADL7 (DMI-R) and GAAP11 (DMI-S).

Fig. 3. Gel electrophoresis analysis of undigested and *BsrBI* digested polymerase chain reaction (PCR) fragments amplified from *Monilinia fructicola* isolate GADL7 with primers *INS65-F* and *INS65-R*. M = *exACTGene* Low Range DNA Ladder (Fisher Scientific); lane 1, undigested PCR fragment; lanes 2 to 6, restriction digests after 0.5, 1, 2, 4, and 16 h of incubation at 37°C, respectively. Digestion was terminated by adding 12.5 mM EDTA followed by a 20-min heat inactivation at 80°C.

digestible with restriction enzyme *BsrBI* (data not shown). One representative sequence (1,815 bp) containing the newly discovered 1,504-bp insertion from DMI-S New York isolate NY3B was submitted to GenBank (accession number EU257287).

RFLP analysis of the PCR fragment amplified with primers *INS65-F* and *INS65-R*. *BsrBI* digestion of the 376-bp PCR fragment generated two restriction fragments of 236 bp and 140 bp in size. Neither the 311-bp nor the 1,815-bp PCR fragment was *BsrBI*-digestible (Table 1; Fig. 1). Efforts to facilitate and shorten the RFLP analysis revealed that the PCR product did not require a purification step prior to endonuclease restriction digestion (data not shown). Also, shortening the incubation time from the manufacturer's recommendation of 16 h to 0.5 h did not result in a noticeable compromise in digestion activity (Fig. 3).

PCR-RFLP analysis was successfully performed using the Lyse-N-GO PCR Reagent (Pierce) with aerial fungal structures obtained from *M. fructicola* cultures and from inoculated fruit as PCR template (Fig. 4). Of the 39 NY cherry isolates grown on PDA, the use of Lyse-N-GO PCR Reagent yielded results 37 times on the first attempt (94.87% success rate; data not shown). Results were obtained for the remaining two isolates on a subsequent attempt (data not shown). Likewise, successful amplifications and restriction digestions were conducted when fungal structures were taken directly from inoculated peach fruit (data not shown).

DISCUSSION

Both point mutations in and the overexpression of the cytochrome P450 lanosterol 14- α demethylase (*CYP51*) gene have been linked to DMI fungicide resis-

tance in plant pathogenic fungi. While point mutations are generally easy to detect with PCR-based molecular techniques (2,3), the detection of *CYP51* overexpression may require a more expensive and elaborate effort such as real-time PCR or northern blot analysis. However, in some plant pathogenic fungi, overexpression is linked to inserts upstream of the *CYP51* gene, which can be used as molecular markers for rapid, PCR-based detection. For example, overexpression of the *CYP51* gene from *P. digitatum* was linked to inserts upstream of *CYP51*, which allowed the development of quick PCR-based detection methods (4,5). Similar inserts were found in isolates of *Venturia inaequalis* and *Blumeriella jaapii* resistant to myclobutanil (13) and fenbuconazole (9), respectively, and PCR-based methods were developed for their identification (11,13).

In this study, we developed a PCR-based assay that distinguishes reliably between *M. fructicola* isolates possessing or lacking the Mona element linked to the DMI-resistance phenotype. The primer pair *INS65-F* and *INS65-R* did not amplify DNA fragments of similar sizes from five other common peach fruit pathogens. This result emphasizes the specificity of this primer pair to the targeted *M. fructicola* DNA. However, the 311-bp and the 376-bp fragments obtained from sensitive and resistant isolates, respectively, may not be easily distinguished on low percentage agarose gels, especially if positive controls are not available. Therefore, the presence or absence of the Mona element in the amplification product can be confirmed by digesting the PCR product with the restriction endonuclease *BsrBI*; only fragments containing the Mona element were digestible with this enzyme. The amplification of a 1,815-bp fragment in DMI-S cherry isolates was somewhat unexpected. However, the fragment neither contained the Mona element nor was digestible by the *BsrBI*

Fig. 4. Gel electrophoresis analysis of undigested and *Bsr*BI digested polymerase chain reaction (PCR) fragments amplified directly from aerial fungal structures of *Monilinia fructicola* DMI-R and DMI-S isolates with primers *INS65-F* and *INS65-R* using the Lyse-N-GO PCR Reagent (Pierce) for template preparation. Lanes 1 and 5, *Quick Load 100-bp Molecular Ladder* (New England Biolabs) and *Quick Load 1kb DNA Ladder* (New England Biolabs), respectively; lanes 2 to 4, undigested PCR fragments from DMI-R isolate NY2F, and DMI-S isolates NY8C and NY3C; lanes 6 to 8, *Bsr*BI digested PCR fragments from DMI-R isolate NY2F, and DMI-S isolates NY8C and NY3C.

endonuclease. Also, the presence of a 1,504-bp insert within the 1,815-bp fragment did not correlate with the DMI resistance phenotype, indicating that it may not influence expression of the *MfCYP51* gene.

The standard PCR-based protocol to detect Mona would normally take several days to conduct because it includes culturing the fungus to obtain sufficient biomass for DNA extraction, PCR amplification, purification of the PCR fragment, restriction digestion, and gel electrophoresis. We found that aerial fungal structures of *M. fructicola* obtained from agar medium and from symptomatic fruit (data not shown) can be used directly as PCR template when lysed in Lyse-N-GO PCR Reagent. We also determined that the PCR product does not need to be purified prior to restriction digestion with the enzyme *Bsr*BI, and that a 30-min restriction digestion is sufficient. Thus, within a few hours, the Mona element can be detected from fungal structures on a symptomatic fruit.

Results of this study confirm that overexpression of the *MfCYP51* gene, as determined by the presence of the Mona element (8), is a common mechanism in conferring DMI resistance in *M. fructicola* in Georgia, South Carolina, New York, and Ohio. This is the first official report of reduced sensitivity to DMI fungicides in *M. fructicola* isolates from South Carolina and Ohio. So far, all DMI-R isolates we collected from the four aforementioned states contained the Mona element upstream of *MfCYP51*. This result is consistent with reports from Japan and California, where DMI resistance in *P. digitatum* was consistently linked to inserts upstream of the *CYP51* gene (4,5). Other

studies have reported strong correlations between inserts and the resistance phenotype, but exceptions were reported where resistant isolates lacked an insert, indicating the existence of other resistance mechanisms (11).

Sequence analysis revealed that the nucleotide composition and the location of the Mona element were identical in isolates from South Carolina, Georgia, New York, and Ohio (data not shown). Therefore, it is likely that all DMI-R isolates derived from a common ancestor. It would therefore be fair to assume that DMI-R isolates are genetically closely related or even identical. In a previous study, DMI-R isolates from Georgia revealed identical nucleotide compositions of the *MfCYP51* gene and the immediate *MfCYP51* upstream region (including the Mona element), whereas DMI-S isolates collected from the same orchards revealed ample nucleotide variations in these regions (8). However, the same study showed genetic variability among DMI-R isolates as detected by microsatellite primer analysis and varying numbers of the Mona element in individual genomes. Based on these results, the authors hypothesized that the insertion event occurred before or shortly after the introduction of DMI fungicides in the 1970s and 1980s. The ubiquity of the Mona element in *M. fructicola* populations stretching from mid-Georgia to New York described in our study supports this hypothesis.

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