atrB-Associated Fludioxonil Resistance in *Botrytis fragariae* Not Linked to Mutations in Transcription Factor *mrr1*

Meng-Jun Hu,¹ Scott Cosseboom,¹ and Guido Schnabel^{2,†}

¹Department of Plant Science and Landscape Architecture, University of Maryland, College Park, MD; and ²Department of Agricultural and Environmental Sciences, Clemson University, Clemson, SC

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ABSTRACT

Resistance to fludioxonil in *Botrytis cinerea* and *B. fragariae* was previously found to be linked to either overexpression of the drug efflux pump *atrB* activated by mutations in transcription factor *mrr1* or to mutations in the osmoregulation gene *osl*. In the present study, isolates of *B. cinerea*, *Botrytis* group S, or *B. fragariae* collected from strawberry fields in the United States were resistant to fludioxonil with half-maximal effective concentration values ranging from 0.04 to 0.43 µg/ml for *B. cinerea*, 0.03 to 1.03 µg/ml for *Botrytis* group S, and 0.28 to 3.48 µg/ml for *B. fragariae*. Analyses of *mrr1* sequences revealed various mutations linked to fludioxonil resistance in *B. cinerea* and *Botrytis* group S isolates. However, no mutations in *mrr1* correlated with *atrB* overexpression-mediated resistance in *B. fragariae* isolates. Neither nucleotide variations in the 1,370-bp upstream region of *atrB* nor

increased *atrB* copy numbers could explain the *atrB* overexpression in these *B. fragariae* isolates. Mutations in *osl* conferred resistance to iprodione in *B. cinerea* and *Botrytis* group S isolates; none correlated with resistance to fludioxonil in *B. fragariae*. In contrast to European isolates, U.S. *B. fragariae* isolates contained a 3-bp insertion in the coding region of *osl*. These isolates were more sensitive to osmotic stress but it is unclear whether the insertion is responsible for this phenotype. Our findings suggest that *atrB* overexpression-associated fludioxonil resistance is an across-species mechanism of resistance to fludioxonil that can be induced by mutations in *mrr1* and other, still-unknown mechanisms.

Keywords: Disease control, disease management, genetics, resistance.

Botrytis fragariae is a newly described fungal pathogen on strawberry (Rupp et al. 2017) and was frequently detected in fields in Germany and the United States (Dowling et al. 2017; Hu et al. 2018; Rupp et al. 2017). *B. fragariae* was reported to be a strawberry-specific pathogen displaying less aggressiveness on nonstrawberry tissues (Rupp et al. 2017). The species is primarily found on strawberry flowers but can also cause gray mold of fruit (Dowling et al. 2017). Our previous studies have shown that 66 of 188 *Botrytis* isolates collected in strawberry fields in the Mid-Atlantic United States were *B. fragariae* (Dowling et al. 2018). Despite distinct infection behavior and genetic background, both *B. cinerea* and *B. fragariae* have developed resistance to multiple chemical classes of fungicides, including the phenylpyrrole (PP) fungicide fludioxonil (Hu et al. 2018; Rupp et al. 2017). In fact, higher frequencies of resistance to fludioxonil were found in *B. fragariae* compared with *B. cinerea* (Dowling et al. 2018).

Resistance to fludioxonil in *B. cinerea* is primarily conferred by overexpression of drug efflux transporters. Kretschmer et al. (2009) first documented *B. cinerea* isolates with resistance to fludioxonil, defined as the MDR1 phenotype. The resistance was found to be associated with mutations in transcription factor *mrr1* causing overexpression of *atrB*, an ATP-binding cassette (ABC) transporter (Kretschmer et al. 2009). Isolates with higher levels of resistance to fludioxonil are classified into *B. cinerea* group S and designated as the MDR1h phenotype. A 3-bp deletion mutation in *mrr1*, leading to an amino acid deletion of L497, was found to be correlated with MDR1h isolates in *Botrytis* group S (Leroch et al. 2012). Except for

mrr1, only mutations in the group III histidine kinase gene *osl* have been reported to be linked to high levels of fludioxonil resistance in *B. cinerea*. Ren et al. (2016) identified mutations in the HAMP domains of the N-terminal region or in the HATPase_c domain of the C-terminal region of *osl* in both field isolates and laboratory mutants. With regard to *B. fragariae*, the mechanism underlying resistance to fludioxonil thus far has been found to be C588Y or R632I mutations in *mrr1*, which are identical to the MDR1-related mutation in *B. cinerea* (Rupp et al. 2017).

Cross-resistance between PPs and dicarboxamides (DCs) is common in *Botrytis* isolates (Faretra and Pollastro 1993; Fillinger et al. 2012; Leroux et al. 1999; Ren et al. 2016), because both chemical classes of fungicides are believed to interfere with osmotic signal transduction. However, this cross-resistance seemed to be inconclusive for MDR1 isolates based on previous studies. Kretschmer et al. (2009) found no significant difference between non-MDR1 and MDR1 isolates in the sensitivity to the DC fungicide iprodione. In contrast, Li et al. (2014) found that all MDR1 isolates resistant to fludioxonil were also resistant to iprodione, indicating the cross-resistance between the two chemical classes. However, iprodione target gene *osl* was not investigated in the MDR1 isolates of the latter study. Cross-resistance in MDR1 isolates between fludioxonil and tolnaftate or fludioxonil and cyprodinil was identified in previous studies (Kretschmer et al. 2009).

The objectives of this study were to determine the sensitivity of *B. fragariae* to fludioxonil and potential cross-resistance to iprodione, cyprodinil, or tolnaftate and to investigate the resistance mechanism associated with fludioxonil resistance.

MATERIALS AND METHODS

Collection, selection, and identification of *Botrytis* spp. isolates. *Botrytis* spp. isolates used in this study were selected from a pool of previously collected *Botrytis* isolates (in 2015 or 2016) from six southeastern United States, including South Carolina,

[†]Corresponding author: G. Schnabel; E-mail: schnabe@clemson.edu

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North Carolina, Georgia, Maryland, Arkansas, and Virginia (Table 1). All isolates were collected from annual strawberry plants that were not subjected to single-site fungicide treatments after planting (except four isolates from fruit) (Table 1). However, the isolates may have been subjected to fungicide pressure at the nursery level. Isolates had been characterized for their fungicide resistance profile (data not shown), as described previously (Fernández-Ortuño et al. 2014; Schnabel et al. 2015). *Botrytis* isolates with low and high levels of fludioxonil resistance, along with some sensitive isolates, were selected for the study. One German *B. fragariae* isolate (D11-H-R4) (Rupp et al. 2017) was kindly provided by Dr. Matthias Hahn at University of Kaiserslautern in Germany. Two *B. cinerea* isolates (106-6-14 and 107-8-14), characterized as MDR1h isolates previously (Chen et al. 2016), were also included in this study. All isolates used in this study were generated from single spores.

Botrytis isolates were grown on potato dextrose agar (PDA) at 22°C for 5 days in darkness. Agar plugs containing mycelia were then transferred to 250-ml flasks containing 50 ml of potato dextrose broth. After 3 days of incubation, mycelium was removed from the broth and subject to DNA extraction using the DNeasy Plant Mini Kit (Qiagen, Valencia, CA, U.S.A.). Species-specific primers (CFM_NEP2, C_NEP2, F_NEP2, and M_NEP2) developed previously (Dowling et al. 2017) were used to identify *Botrytis* spp. isolates collected from strawberry to the species level. Polymerase chain reactions (PCRs) were carried out in a 25-μl system containing 10 ng of template DNA, 0.4 μM each primer, 200 μM dNTP, 1× PCR buffer (New England Biolabs), and 1 U of Taq DNA polymerase (New England Biolabs). The amplification program consisted of an initial denaturation at 94°C for 3 min; followed by 34 cycles of 94°C for 40 s, annealing at 55°C for 40 s, and extension at 72°C for 1 min; and a final extension of 72°C for 10 min. PCR products were separated on a 1% (wt/vol) agarose gel in 0.5% Tris-acetate-EDTA buffer for 30 min at 100 V. The gel was then stained with GelRed (Biotium Inc., Fremont, CA, U.S.A.) and exposed to UV light to visualize DNA fragments.

In vitro fungicide sensitivity assays. Sensitivity of *Botrytis* spp. isolates to fludioxonil (Scholar; Syngenta, Greensboro, NC, U.S.A.), cyprodinil (Vangard; Syngenta), iprodione (Rovral; Bayer Crop Science, Research Triangle Park, NC, U.S.A.), or tolnaftate (Sigma-Aldrich, St. Louis, MO, U.S.A.) was determined using a spore-based microtiter assay (Stammler and Speakman 2006). To make conidial suspensions, isolates were grown on PDA (Difco) plates at 22°C for 5 days in darkness, followed by 1 week of incubation under light conditions. Spores were then harvested in

3 ml of double concentrated malt extract broth (2× MEB) by gently scratching the colonies using a sterilized cotton swab. The resulting suspensions were filtered with two-layer cloth and diluted to 5×10^4 spores/ml. Final fungicidal concentrations of 0, 0.1, 0.3, 1, 3, 10, 30, and 100 μg/ml were used in microtiter assays for fludioxonil, cyprodinil, and iprodione, while 0, 0.03, 0.1, 0.3, 1, 3, 10, and 30 μg/ml were used for tolnaftate. Fungicide solutions were prepared in sterilized water and each solution (50 μl) was transferred into 96-well microtiter plates and mixed with an equal volume (50 μl) of spore suspension, as described previously (Hu et al. 2015). Each isolate and fungicide concentration had three replicates. After 2 days of incubation at 22°C in darkness, absorbance of 600 nm was determined with a photometer. A linear regression analysis was performed to calculate the half-maximal effective concentration (EC₅₀) values for each isolate. In case a poor correlation coefficient was observed, the experiment was then repeated for that isolate.

In addition to determining the EC₅₀ as described above, the 90% mycelial growth inhibition (EC₉₀) value was determined for fludioxonil. Briefly, isolates were grown on malt extract agar media amended with fludioxonil at 0, 3, 10, and 100 μg/ml. After 1 week of incubation at 22°C in the dark, mycelial growth inhibition ≥90% was measured. EC₉₀ value was expressed as greater or smaller than 3, 10, or 100 μg/ml for each isolate.

Detached-fruit assay. A selection of *B. cinerea*, *Botrytis* group S, and *B. fragariae* isolates (nine in total) with various sensitivities to fludioxonil were chosen to be tested for practical resistance to fludioxonil (Table 2). Store-bought, organic strawberry fruit were treated with the recommended label rate (410 μg/ml) of fludioxonil using the formulated product Scholar (Syngenta). The diluted fungicide was applied to strawberry fruit using a spray bottle until runoff; then, the fruit were allowed to dry for 2 h. The strawberry fruit were then wounded by inserting a sterile toothpick to a depth of 1 cm into the receptacle of each fruit. Because some isolates did not easily sporulate, mycelia of each isolate were transferred into the wound for seven fruit per isolate. Seven other strawberry fruit were treated with fludioxonil and wounded but were not inoculated. The fruit were incubated in humid chambers for 5 days, and then incidence of gray mold and lesion diameter was evaluated for each fruit. This experiment was conducted twice. A Kruskal-Wallis test was used to test the effect of isolate on lesion diameter, and was followed by a Dunn's multiple comparisons test.

Sequencing of *mrr1* and *os1* in *Botrytis* spp. isolates. *Mrr1* and *os1* genes were amplified and sequenced from *B. cinerea* and *B. fragariae* in order to investigate the molecular basis of resistance to fludioxonil. Primer information, including name, nucleotide

TABLE 1. Strawberry *Botrytis* spp. isolates used in this study and their characteristics regarding the sensitivity to fludioxonil

Isolates	EC ₅₀	EC ₉₀ ^x	Sensitivity ^y	AA alteration in <i>mrr1</i>	Species	Phenotype	Host tissue	Location
4-8-15	0.04	<3	S	None	<i>Botrytis cinerea</i>	Non-MDR	Fruit	Anderson, SC
27-5-16	0.43	<3	LR	R351C	<i>B. cinerea</i>	MDR1	Flower	Lancaster, SC
5d5	0.03	<3	S	T449S/G702N	<i>Botrytis</i> group S	Non-MDR	Flower	Spartanburg, SC
6-3-15	0.29	<3	LR	D354Y/G702N	<i>Botrytis</i> group S	MDR1	Fruit	Chesterfield, SC
18-3-16	1.03	<3	MR	ΔL/V497	<i>Botrytis</i> group S	MDR1h	Flower	Duplin, NC
106-6-14	1.32	>100	MR	ΔL/V497	<i>Botrytis</i> group S	MDR1h	Fruit	Tolland, CT
107-8-14	2.22	>100	MR	ΔL/V497	<i>Botrytis</i> group S	MDR1h	Flower	Tolland, CT
D11-H-R4 ^z	0.22	<3	S	None	<i>B. fragariae</i>	Non-MDR	Flower	Germany
12-3-16	0.28	<3	S	None	<i>B. fragariae</i>	Non-MDR	Flower	York, SC
11-1-16	0.32	<3	S	None	<i>B. fragariae</i>	Non-MDR	Fruit	Colquitt, GA
56-6-16	0.49	n.d.	S	None	<i>B. fragariae</i>	Unknown	Flower	Calvert, MD
21-8-16	0.88	>30	LR	None	<i>B. fragariae</i>	Unknown	Flower	Charleston, SC
77-6-16	1.31	>100	MR	None	<i>B. fragariae</i>	Unknown	Flower	Harford, MD
27-2-16	1.90	>100	MR	None	<i>B. fragariae</i>	Unknown	Flower	Lancaster, SC
7-3-15	2.38	>100	MR	n.d.	<i>B. fragariae</i>	Unknown	Flower	Desha, AR
27-9-16	3.04	>100	MR	None	<i>B. fragariae</i>	Unknown	Flower	Lancaster, SC
52-7-16	3.11	>100	MR	None	<i>B. fragariae</i>	Unknown	Flower	Virginia Beach, VA
31-10-16	3.37	>100	MR	None	<i>B. fragariae</i>	Unknown	Flower	Virginia Beach, VA
79-5-16	3.48	>100	MR	None	<i>B. fragariae</i>	Unknown	Flower	Hanover, VA

^x Abbreviation n.d. = not determined.

^y Abbreviations S, LR, and MR stand for sensitive, low resistance, and medium resistance, respectively.

^z Isolate was collected in strawberry field in Germany.

sequence, description, and reference, is shown in Table 3. PCRs were performed in a volume of 25 µl containing 10 ng of DNA template, 0.4 µM each primer, 200 µM dNTP, 1× PCR buffer (New England Biolabs), and 1 U of Taq DNA polymerase (New England Biolabs). A PCR program of 94°C for 3 min; followed by 34 cycles of 94°C for 40 s, 55°C for 40 s, and extension at 72°C for 1 min and 30 s; and a final step of 72°C for 10 min was used for the amplification of both *mrr1* and *os1*. PCR products were verified by electrophoresis as described above and sent to the DNA laboratory at Arizona State University in Tempe, AZ. The *mrr1* sequences of *B. cinerea* isolate N11_K_W08b (GenBank accession number JX266761), *Botrytis* group S isolate D10_B_F1106 (JX266766), and *B. fragariae* isolate D11-H-R4 (KX429733) and *os1* sequences of *B. cinerea* (AF435964) and *B. fragariae* isolate D13_D_F_Ju21 (KX429732) were used as reference sequences for identifying potential nucleotide mutations.

Osmotic stress assays and detection of 3-bp insertion in *os1* of *B. fragariae*. To determine the sensitivity of *Botrytis* spp. isolates to osmotic stress, a mycelial plug (5 mm in diameter) taken from a 4-day old culture grown on PDA was placed on minimal media [MM; (Hu et al. 2011)] and PDA media amended with 4% NaCl. Petri dishes were incubated for 5 days at 22°C in darkness before taking measurements of the colony diameter. The susceptibility to osmotic stress was evaluated by calculating the mycelial growth inhibition compared with the control. Each isolate had three replicates and the experiment was conducted once.

To investigate the association between the 3-bp insertions in *os1* and reduced sensitivity to osmotic stress in the US *B. fragariae* isolates, primer pair *os1* 3bp-R and BF1_56_opt (Table 3) was used to detect the 3-bp insertion. The PCR amplification program was identical with that for *mrr1* and *os1* amplification, except the extension duration was 30 s instead of 1 min.

TABLE 2. Lesion diameter and infection frequency of fludioxonil-treated strawberry fruit inoculated with different phenotypes of *Botrytis cinerea*, *Botrytis* group S, and *Botrytis fragariae* from two experiments

Isolate	Phenotype	Sensitivity ^z	Species	Infection frequency (%)		Average lesion diameter (mm) ^y	
				Exp. 1	Exp. 2	Exp. 1	Exp. 2
4-8-15	Non-MDR	S	<i>B. cinerea</i>	0	29	0.0 b	0.4 bc
12-3-16	Non-MDR	S	<i>B. fragariae</i>	29	14	7.9 ab	0.3 c
11-1-16	Non-MDR	S	<i>B. fragariae</i>	0	14	0.0 b	0.3 c
5d5	Non-MDR	S	<i>Botrytis</i> group S	0	0	0.0 b	0.0 c
27-5-16	MDR1	LR	<i>B. cinerea</i>	100	86	12.7 ab	4.9 abc
21-8-16	Unknown	LR	<i>B. fragariae</i>	100	71	13.7 a	2.1 abc
27-9-16	Unknown	MR	<i>B. fragariae</i>	100	100	15.6 a	6.4 ab
31-10-16	Unknown	MR	<i>B. fragariae</i>	100	100	10.0 ab	6.4 ab
107-8-3	MDR1h	MR	<i>Botrytis</i> group S	86	100	17.3 a	25.0 a

^y Lesion diameters with the same letters are not significantly different ($P < 0.05$) according to a Dunn's multiple comparisons test.

^z Abbreviations S, LR, and MR stand for sensitive, low resistance, and medium resistance, respectively, based on half-maximal effective concentration and 90% mycelial growth inhibition values.

TABLE 3. Primers used in the study

Primer name	Nucleotide sequence (5' → 3')	Gene	Description	Reference
<i>atrB</i> _RT_for2	ACGTTTGACAATTGGTGTGG	<i>atrB</i>	Real-time polymerase chain reaction (PCR) primers for <i>Botrytis fragariae</i> and <i>B. cinerea</i>	Present study
<i>atrB</i> _RT_rev2	GAGTTGAGCGGAAGGTTGAT	<i>atrB</i>	Same as <i>atrB</i> _RT_for2	Present study
<i>atrB</i> _5UTR_F	CGGTGTAGCATACCGTCTC	<i>atrB</i>	To amplify and sequence upstream of <i>atrB</i> in <i>B. fragariae</i>	Present study
<i>atrB</i> _5UTR_R	CATGTCCTTCGTGTGGTCGA	<i>atrB</i>	same as <i>atrB</i> _5UTR_F	Present study
BcAct_RTfor	TCTGTCTTGGGTCTTGAGAG	<i>actin</i>	Real-time PCR primers for <i>B. fragariae</i> and <i>B. cinerea</i>	Kretschmer et al. (2009)
BcAct_RTrev	GGTGCAAGAGCAGTGATTTC	<i>actin</i>	Same as BcAct_RTfor	Kretschmer et al. (2009)
Mrr1-atg	TCAACATCATGAATCCAACAGTC	<i>mrr1</i>	To amplify and sequence <i>mrr1</i> in <i>B. cinerea</i> and <i>B. fragariae</i>	Kretschmer et al. (2009)
TF1-2-new	CTATCCGATCGACCGGTA	<i>mrr1</i>	To amplify and sequence <i>mrr1</i> in <i>B. cinerea</i>	Leroch et al. (2012)
BcinN-in-R	GGCTCTCGATGAGCTGTTTC	<i>mrr1</i>	To amplify and sequence <i>mrr1</i> in <i>B. fragariae</i>	Plesken et al. (2015)
Bf_Mrr1_F	ATCAAACGCCAAGAGACCGAG	<i>mrr1</i>	Same as BcinN-in-R	Present study
Bf_Mrr1_R	TATCAAAAGAGCCATCAGTG	<i>mrr1</i>	Same as BcinN-in-R	Present study
TF1-3new	TGCTGTGACGAGCATGAC	<i>mrr1</i>	Same as Mrr1-atg	Leroch et al. (2012)
TF1-4	GGATAGGGTATTGCGTAGATCG	<i>mrr1</i>	Same as Mrr1-atg	Kretschmer et al. (2009)
BF1_56_opt	TTAGCAGCCTGGCTGCATATAC	<i>os1</i>	To amplify and sequence <i>os1</i> in <i>B. cinerea</i> and <i>B. fragariae</i> , to detect the 3bp insertion in <i>B. fragariae</i>	Rupp et al. (2017)
BR1_1084_opt	TTGTGCCATGACGTTGAC	<i>os1</i>	To amplify and sequence <i>os1</i> in <i>B. cinerea</i> and <i>B. fragariae</i>	Rupp et al. (2017)
BF2	CAACGTTATGGCAGAAATCTCA	<i>os1</i>	Same as BR1_1084_opt	Ma et al. (2007)
BR2	AAGTTTCTGGCCATGGTGTCA	<i>os1</i>	Same as BR1_1084_opt	Ma et al. (2007)
BF3_opt	TCGAGGTTTCAGGGAGAAATC	<i>os1</i>	Same as BR1_1084_opt	Rupp et al. (2017)
BR3_opt	CTATCCACGCGGTAAGTGAG	<i>os1</i>	Same as BR1_1084_opt	Rupp et al. (2017)
BF4	GCAAACCGTATGATCATGGA	<i>os1</i>	Same as BR1_1084_opt	Ma et al. (2007)
BR4_opt	TCTTCGGCCAAGAGAATGTC	<i>os1</i>	Same as BR1_1084_opt	Rupp et al. (2017)
BF5_opt1	TCGATGAGTTCAAGTATATC	<i>os1</i>	Same as BR1_1084_opt	Present study
BR5	AAGTACTCGCAGTCGGTGGT	<i>os1</i>	Same as BR1_1084_opt	Ma et al. (2007)
Bos1 3bp-R	TGTTGTCGAGATGTAGTACTAC	<i>os1</i>	To detect the 3-bp insertion in <i>B. fragariae</i>	Present study

Relative expression analysis of *atrB*. The relative expression of *atrB* was determined in *B. fragariae* isolates with and without resistance to fludioxonil as well as MDR1h isolates of *Botrytis* group S. Single mycelial plugs were taken from a 4-day-old culture grown on PDA and transferred to flasks containing 50 ml of MEB. Flasks were then placed in a shaker at 120 rpm at room temperature for 4 days. Trizol reagent (Invitrogen, Carlsbad, CA, U.S.A.) was used to extract total RNA from mycelia following the manufacturer's protocol. First-strand cDNA was then synthesized from the resulting RNA using a Bio-Rad iScript cDNA kit (Bio-Rad, Hercules, CA, U.S.A.).

For quantitative PCR analysis, primer pairs *atrB_RT_for2/atrB_RT_rev2* and *BcAct_RTfor/BcAct_RTrev* (Table 3) were designed to amplify the target gene *atrB* and reference gene *actin*, respectively. The PCR was conducted using iQ SYBR Green Supermix (Bio-Rad) according to the manufacturer's instructions. The amplification programs consisted of an initial denaturation at 95°C for 2 min, followed by 45 cycles at 95°C for 15 s, 57°C for 15 s, and 72°C for 15 s. A melting curve analysis was also performed to verify the specificity of amplified products. Three replicates were used for each sample. Two experiments with new biological replicates were conducted independently. Analysis of significant difference procedures was conducted using JMP Pro 13.1.0 software at $P = 0.05$ (SAS Institute Inc., Cary, NC, U.S.A.).

Investigations of upstream sequences and copy number of *atrB*. Based on the 5' flanking sequences of *atrB* obtained from the genome-sequenced *B. cinerea* isolate B05.10, primers *atrB_5UTR_F* and *atrB_5UTR_R* (Table 3) were designed to amplify and sequence upstream sequences of *atrB* from *B. fragariae*. PCR amplification protocol was identical with that for *mrr1* and *os1* amplification, except for a 2-min extension at 72°C. PCR products were verified by electrophoresis as described above and sent to the DNA laboratory at Arizona State University in Tempe, AZ. Benchling (<https://benchling.com/>) was used for DNA sequence analysis. Promoter predictor software NNPP (http://www.fruitfly.org/seq_tools/promoter.html) was used to analyze promoter sequences.

To investigate the copy number of *atrB* in *Botrytis* spp. isolates with and without resistance to fludioxonil, the same primers used for relative expression analysis of *atrB* were used to amplify the *atrB* and *actin* genes from genomic DNA (Table 3). The PCR was conducted using iQ SYBR Green Supermix (Bio-Rad) according to the manufacturer's instructions. The amplification programs were identical with those of quantitative PCR analysis described above.

RESULTS

Identification of *Botrytis* spp. isolates from strawberry fields selected for the study. Of the 16 *Botrytis* isolates in this

study, excluding those characterized previously (106-6-14, 107-8-14, and D11-H-R4), 5 were identified to be *B. cinerea* and 11 were *B. fragariae* (Table 1). Both species were collected primarily from blossoms but also from some fruit. *B. fragariae* isolates were found in South Carolina, Georgia, Maryland, Virginia, and Arkansas (Table 1).

Determination of sensitivity to fludioxonil of *B. cinerea*, *Botrytis* group S, and *B. fragariae* isolates. A spore-based microtiter assay determined fludioxonil sensitivity in all isolates. Overall, the EC_{50} values ranged from 0.04 to 3.48 $\mu\text{g/ml}$ (Table 1). Based on the EC_{50} values and resistance categories described previously (Fernández-Ortuño et al. 2015), *B. cinerea* and *Botrytis* group S isolates with EC_{50} values ranging from 0.01 to 0.1, 0.1 to 0.5, and 0.5 to 3 $\mu\text{g/ml}$ were considered as sensitive (S), low resistant (LR), and moderately resistant (MR), respectively. Rupp et al. (2017) found that EC_{50} values of three fludioxonil-sensitive *B. fragariae* isolates ranged from 0.18 to 0.24 $\mu\text{g/ml}$, which were higher than those of fludioxonil-sensitive *B. cinerea* isolates. Thus, *B. fragariae* isolates with EC_{50} values of 0.1 to 0.5, 0.5 to 1.0, and 1.0 to 5.0 $\mu\text{g/ml}$ were considered as S, LR, and MR, respectively (Table 1). EC_{90} values are shown as <3, >30 but >100, and >100 $\mu\text{g/ml}$. The EC_{90} values of the majority of S and LR isolates were <3 $\mu\text{g/ml}$. All MR isolates, except 18-3-16, had EC_{90} values > 100 $\mu\text{g/ml}$.

Detached-strawberry-fruit assays. The detached-fruit assay revealed that *Botrytis* isolates considered LR and MR tended to cause gray mold in higher frequencies, ranging from 71 to 100 and 0 to 29% for resistant (R, including LR and MR) and S isolates respectively (Table 2). No lesions formed on the noninoculated control fruit. Experiments 1 and 2 yielded similar results, and a significant effect of inoculum on the lesion diameter was detected in both the first ($H = 39.420$, $P < 0.001$) and second ($H = 50.626$, $P < 0.0001$)

B. fragariae (Germany)

Fig. 1. Schematic of *os1* gene exon and intron organizations in *Botrytis fragariae* from Germany and the United States. Exons and introns are represented by dark rectangles and solid lines, respectively. The “Y” symbol indicates a position where an insertion event occurred.

TABLE 4. Sensitivity to cyprodinil, tolnaftate, and iprodione, and *os1* mutations in *Botrytis* spp. isolates with different phenotypes

Isolate	Sensitivity (EC_{50}) ^y			AA alteration in <i>os1</i>	Species	Phenotype
	Cyprodinil	Tolnaftate	Iprodione			
4-8-15	0.099	1.13	0.49	None	<i>Botrytis cinerea</i>	Non-MDR
27-5-16	0.404	>10	>100	I365S/D757 N/A1259T	<i>B. cinerea</i>	MDR1
5d5	2.47	1.62	4.66	F127S/I365N	<i>Botrytis</i> group S	Non-MDR
6-3-15	0.267	>10	>11.22	n.d.	<i>Botrytis</i> group S	MDR1
18-3-16	0.102	4.31	>100	F127S/I365N	<i>Botrytis</i> group S	MDR1h
106-6-14	>10	>10	>100	I365N	<i>Botrytis</i> group S	MDR1h
107-8-14	0.376	n.d.	>100	I365S/A871V/A1259T	<i>Botrytis</i> group S	MDR1h
D11-H-R4 ^z	>10	>10	>100	G367A/V368F	<i>B. fragariae</i>	Non-MDR
11-1-16	n.d.	0.13	n.d.	Insertion S101	<i>B. fragariae</i>	Non-MDR
12-3-16	n.d.	0.21	n.d.	Insertion S101	<i>B. fragariae</i>	Non-MDR
21-8-16	0.084	>10	1.75	Insertion S101	<i>B. fragariae</i>	Unknown
77-6-16	0.089	>10	1.83	Insertion S101	<i>B. fragariae</i>	Unknown
7-3-15	0.103	>10	3.36	Insertion S101	<i>B. fragariae</i>	Unknown
31-10-16	1.376	n.d.	8.34	n.d.	<i>B. fragariae</i>	Unknown

^y EC_{50} = half-maximal effective concentration; n.d. = not determined.

^z Isolate was collected in Germany.

assays. Isolates considered LR and MR tended to have greater lesion diameters compared with the S isolates in both assays, with no exceptions (Table 2). Significant differences in lesion diameters were detected between some R and S isolates. For example, the lesion diameter of *B. fragariae* MR isolate 27-9-16 was significantly larger than all of the S isolates in both assays (Table 2).

Sequencing analysis of *mrr1* in *Botrytis* spp. isolates. The entire *mrr1* gene, approximately 2,450 bp, was successfully amplified from all *B. cinerea* and *B. fragariae* isolates with seven primers (Table 3). Based on the *mrr1* sequences, five *B. cinerea* isolates were further identified as *Botrytis* group S (Table 1) according to Leroch et al. (2012). No nucleotide mutations were found in *mrr1* from the *B. cinerea* sensitive isolate (4-8-15), while amino acid substitutions of serine (S) for threonine (T) and asparagine (N) for glycine (G) were found at positions 449 and 702, respectively, in the other fludioxonil-sensitive isolate (group S; 5d5). D354Y and G702 N were detected in one *B. cinerea* LR isolate (6-3-15), whereas the other LR isolate (27-5-16) had the R351C mutation. All *B. cinerea* MR isolates belonging to *Botrytis* group S possessed the L497 deletion in *mrr1*, indicating them to be MDR1h (Leroch et al. 2012). None of the *B. fragariae* isolates examined had mutations in the *mrr1* gene (Table 1). The *mrr1* sequences of *B. fragariae* isolate 79-5-16; *Botrytis* group S isolates 18-3-16, 5d5, and 6-3-15; and *B. cinerea* isolates 27-5-16 and 4-8-15 were deposited in GenBank under accession numbers MH721887, MH721889, MH721890, MH721891, MH721888, and MH721892, respectively.

Sensitivity to iprodione, cyprodinil, and tolnaftate and sequencing analysis of *os1*. In order to investigate cross-resistance patterns between PPs (e.g., fludioxonil) and DCs (e.g., iprodione), and between PPs and anilinopyrimidines (e.g., cyprodinil), the sensitivity to iprodione and cyprodinil was determined using microtiter assays. Tolnaftate was also included in the sensitivity assays because resistance to this product indicates increased drug efflux pump activity in MDR phenotypes (Kretschmer et al. 2009). No cross-resistance was observed between fludioxonil and cyprodinil. For example, *B. cinerea*

isolates 6-3-15 and 18-3-16 resistant to fludioxonil were both sensitive to cyprodinil (Table 4). EC₅₀ values of all MDR isolates, including MDR1 and MDR1h, ranged from 4.31 to >10 µg/ml for tolnaftate, whereas EC₅₀ values of non-MDR isolates ranged from 0.13 to 1.62 µg/ml (Table 4). For iprodione, EC₅₀ of *B. cinerea* isolates (including group S) and *B. fragariae* isolates ranged from 0.49 to >100 µg/ml and 1.75 to >100 µg/ml, respectively.

The entire *os1* gene sequence was successfully cloned from *B. cinerea* and *B. fragariae* isolates. The gene was approximately 4,472 bp in length for both species and consisted of 5 introns and 6 exons (Fig. 1). The analysis of *os1* sequences revealed that iprodione resistance was linked to a variety of mutations. For example, I365S, D757 N, and A1259T were found in *B. cinerea* isolate 27-5-16, whereas F127S and I365 N were found in the *Botrytis* group S isolate 5d5. All of the isolates resistant to iprodione, except 106-6, contained two or more mutations in *os1* (Table 4). A 3-bp insertion was found at position 101 of *os1*, leading to a serine insertion in some fludioxonil-sensitive and -resistant isolates of *B. fragariae* (Fig. 1; Table 4). The *os1* sequence of *B. fragariae* isolate 27-9-16 was deposited in GenBank under accession number MH721893.

Sensitivity of *Botrytis* spp. isolates to NaCl and investigation of the 3-bp insertion in *os1*. Except for the German isolate (D11-H-R4), mycelial growth of *B. fragariae* isolates was inhibited more by NaCl-amended media compared with *B. cinerea* isolates (Fig. 2). On MM, the inhibition rate of the U.S. *B. fragariae* isolates ranged from 62.5 to 67.6%, whereas those of *B. cinerea* isolates ranged from 20.0 to 48.8%. Similarly, the inhibition rate of the U.S. *B. fragariae* and *B. cinerea* isolates (including *Botrytis* group S) ranged from 61.3 to 71.8 and 16.8 to 41.1%, respectively, on PDA (Fig. 2). PCR primer pair *os1* 3bp-R and BF1_56_opt detected the 3-bp insertion in all U.S. *B. fragariae* isolates (Table 1) but not in the German isolate D11-H-R4 (Fig. 1). In addition, the 3-bp insertion was absent in all *B. cinerea* isolates tested in the study (Table 4).

Relative expression of *atrB* in *Botrytis* spp. isolates with and without resistance to fludioxonil. Quantitative real-time PCR was performed to investigate whether *atrB* was differentially

Fig. 2. Sensitivity of *Botrytis cinerea*, *B. fragariae*, and *Botrytis* group S isolates to osmotic stress (4% NaCl). Dark and white bars indicate mycelium growth inhibition (%) of individual isolates grown on minimal medium (MM) and potato dextrose agar (PDA), respectively.

expressed in *B. fragariae* isolates sensitive or resistant to fludioxonil. *Botrytis* group S MDR1h isolates 106-6-14 and 107-8-14 were included in the experiments to enable a direct comparison between two species. The mean values of relative expression ranged from 1.3 to 2.9, 15.3 to 21.3, and 4.4 to 5.1 for *B. fragariae* sensitive isolates, *B. fragariae* resistant isolates, and *B. cinerea* MDR1h isolates, respectively (Fig. 3). The highest expression level of *atrB* was found in *B. fragariae* resistant isolates compared with *B. fragariae* sensitive isolates and MDR1h isolates. *B. fragariae* sensitive isolate D11-H-R4 had the lowest relative expression of *atrB*, while the highest expression was detected in the resistant isolate 7-3-15. A significantly higher ($P < 0.05$) expression level of *atrB* was found in MDR1h isolates 106-6-14 and 107-8-14 than in *B. fragariae* sensitive isolates (D11-H-R4, 11-1-16, and 12-3-16).

Analyses of upstream sequences and copy number of *atrB*. In an attempt to explain the molecular basis of the *atrB* overexpression in *B. fragariae* isolates resistant to fludioxonil, the upstream sequence was searched for variations and the copy number of the *atrB* gene was investigated. The 1,370-bp upstream sequences were identical between four resistant and four sensitive *B. fragariae* isolates, except for a 41-bp insertion (5'CGGAGATAGGTGAATTTCGAACACATTAGATCACTCACGGCG3') in one resistant isolate (77-6-16). The insertion was located 1,050 bases upstream of the start codon of *atrB*. The insertion did not contain predicted promoter sequences. The results of quantitative PCR showed less than twofold difference in mean value of relative copy number among five isolates tested, ranging from 1.2 to 1.9 (Fig. 4). The *atrB* upstream sequences of *B. fragariae* isolates 31-10-16, 27-2-16, 12-3-16, 11-1-16, and 77-6-16 were deposited in GenBank under accession numbers MH721882, MH721883, MH721884, MH721885, and MH721886, respectively.

DISCUSSION

Two mechanisms conferring resistance to fludioxonil in *B. cinerea* have been reported. The first and most common is the overexpression of *atrB*, leading to active removal of the fungicide from of the cell with this membrane-bound ABC transporter. The overexpression has always been linked to *mrr1* mutations in both *B. cinerea* and *B. fragariae* from Europe and the United States (Fernández-Ortuño et al. 2015; Kretschmer et al. 2009; Li et al. 2014; Rupp et al. 2017). This mechanism of resistance is associated with EC₅₀ levels ranging from 0.16 to 0.4 µg/ml for MDR1 isolates and 0.75 to 3.1 µg/ml for MDR1h isolates of *B. cinerea* and *Botrytis* group S (Fernández-Ortuño et al. 2015; Rupp et al. 2017), and from

0.86 to 1.0 µg/ml for MDR1 isolates of *B. fragariae* (Rupp et al. 2017). The other mechanism is based on mutations in *os1* leading to high levels of resistance to fludioxonil (EC₅₀ values > 100 µg/ml) in *B. cinerea* field isolates from China, and the mutations were located in the HAMP domains of the N-terminal region (Ren et al. 2016). In agreement with the previous studies, the EC₅₀ values of MDR1 or MDR1h isolates fell into the same range of each phenotype (Table 1), allowing for a valid comparison of EC₅₀ values of U.S. *B. fragariae* isolates with other resistant phenotypes in this study.

Overexpression of target genes or ABC transporters has been found to confer fungicide resistance in many fungal pathogens. In most cases, the overexpression was caused by rearrangement of upstream promoters or mutations in transcription factors, which constitutively regulate gene expression (Kretschmer et al. 2009; Luo and Schnabel 2008; Schnabel and Jones 2001). In this study, the *atrB* gene was constitutively expressed in all untreated *B. fragariae* isolates with resistance to fludioxonil. Only minor differences were found between fludioxonil-treated and -untreated *B. fragariae* resistant isolates in *atrB* expression (data not shown). Although a 41-bp insertion was present at position 1,050 bp of the *atrB* upstream region in the resistant isolate 77-6-16, it was absent in other resistant isolates in which *atrB* was consistently highly expressed. The presence of the insertion did not alter upstream promoter sequences of *atrB* according to NNPP prediction, nor did the insertion contain a promoter sequence (data not shown). Final proof of involvement for fludioxonil resistance was that the same 41-bp insertion was found in a *B. fragariae* sensitive isolate (data not shown), which further confirmed the disassociation with the *atrB* overexpression.

Gene duplication and amplification is considered an important adaptive mechanism in bacteria in response to the presence of antibiotics (Sandegren and Andersson 2009). An increase in gene copy number can be responsible for rapid evolution of resistance of plants to herbicides (Koo et al. 2018). However, this mechanism has not been correlated with fungicide resistance in plant pathogens and our results also did not support gene duplication as a viable mechanism for resistance to fludioxonil. Therefore, the molecular basis for the high level of *atrB* expression in our resistant *B. fragariae* isolates remains elusive but could be linked to a cryptic transcription factor different from *mrr1*. A previous study has shown that R632I mutation in *mrr1* was not detected in lab mutants of *B. cinerea* with EC₅₀ values > 50 µg/ml for fludioxonil, indicating that the fungus may be using additional mechanisms to mediate resistance (Jurick et al. 2017). However, other possible MDR1-related mutations or MDR1h's deletion in *mrr1* were not

Fig. 3. Relative expression of the *atrB* gene in *Botrytis* spp. isolates with differential sensitivity to fludioxonil. The *atrB* expression was normalized with the *actin* gene expression and then calibrated to the values of mRNA amount based on the isolate with the lowest amount of *atrB* mRNA.

Fig. 4. Relative copy number of the *atrB* gene in *Botrytis* spp. isolates with differential sensitivity to fludioxonil. The *atrB* copy number was normalized with the *actin* gene copy number and then calibrated to the values of genomic DNA (gDNA) amount based on the isolate with the lowest amount of *atrB* gDNA.

investigated in the same study. In fact, a high level of resistance to fludioxonil in *B. cinerea* is usually correlated with *os1* mutations and none of the mutations in *mrr1* revealed thus far can lead to EC₅₀ values > 50 µg/ml (see above for details).

Positive correlations have been observed between the level of fludioxonil resistance and *atrB* expression. *atrB* is up to 3-fold more expressed in MDR1h isolates compared with MDR1 isolates and >100-fold more expressed than in *B. cinerea* sensitive isolates (Fernández-Ortuño et al. 2015; Li et al. 2014). *B. fragariae* isolates tended to have higher levels of *atrB* expression, resulting in an intrinsically lower sensitivity to fludioxonil compared with *B. cinerea* and *Botrytis* group S (Rupp et al. 2017). For example, similar levels of *atrB* expression were found in *B. fragariae* MDR1 isolates and *Botrytis* group S MDR1h isolates. In our study, *atrB* expression of fludioxonil-sensitive *B. fragariae* isolates was only slightly lower than in *Botrytis* group S MDR1h isolates, which is consistent with the previous study (Rupp et al. 2017). Interestingly, the *atrB* expression of our fludioxonil MR *B. fragariae* isolates was approximately fourfold higher, on average, than *Botrytis* group S MDR1h isolates, and revealed the highest resistance levels (EC₅₀ range from 0.88 to 3.48 µg/ml) of all resistant phenotypes identified in Europe or the United States thus far.

Partial resistance to cyprodinil has been associated with fludioxonil resistance in *Botrytis* MDR1 and MDR1h isolates (Kretschmer et al. 2009; Leroy et al. 2012). However, in our study, *B. fragariae* isolates with resistance to fludioxonil were sensitive to cyprodinil. Thus, at least in our isolates, *atrB* was not a genetic determinant of partial resistance to cyprodinil. On the other hand, resistance to toltafate was present in the fludioxonil-resistant *B. fragariae* isolates, confirming that *atrB* overexpression confers resistance to toltafate in both *B. cinerea* and *B. fragariae*. Although slightly lower sensitivity to iprodione was observed in those *B. fragariae* isolates compared with *B. cinerea* isolate 4-8-1, no clear evidence was found to support cross-resistance between iprodione and fludioxonil. Rupp et al. (2017) showed that the EC₅₀ values of four European *B. fragariae* sensitive isolates ranged from 0.79 to 1.95 µg/ml for iprodione, which were lower than but comparable with those of our *B. fragariae* isolates. The slightly higher tolerance to iprodione in the U.S. *B. fragariae* could be due to the 3-bp insertion in *os1*. Iprodione resistance found in all of the MDR1 and MDR1h isolates was correlated with mutations in *os1*, indicating that *atrB* overexpression in *Botrytis* isolates may not confer resistance to DCs such as iprodione.

Increased sensitivity to osmotic stress has been found in *Botrytis* isolates with moderate resistance to fludioxonil but not in isolates with low resistance (Li et al. 2014). A similar pattern was found among *B. cinerea* and *Botrytis* group S isolates in our study. It is interesting that all *B. fragariae* isolates collected in the United States, including both fludioxonil-sensitive and -resistant isolates, were highly sensitive to 4% NaCl, indicating that osmotic sensitivity remained unaffected by the overexpression of *atrB* in the resistant *B. fragariae* isolates. Increased sensitivity to osmotic stress has been linked to mutations in osmoregulation genes such as *os1* (Ren et al. 2016) and *os2* (Zhang et al. 2002). Whether the 3-bp insertion in the *os1* coding region found in all U.S. *B. fragariae* isolates was responsible for the increased osmotic sensitivity is unknown. Investigation of osmotic stress in more European *B. fragariae* isolates will further reveal the role of the 3-bp insertion. In addition, the 3-bp insertion may serve as a molecular marker for U.S. *B. fragariae*. A variety of factors, including mutation, gene migration, selection, population size, and mating system, can contribute to genetic change within a population (McDonald 1997). A genetic analysis of European and U.S. *B. fragariae* populations may further confirm their genetic differences and reveal genetic structure within and among populations.

A follow-up experiment was conducted to investigate potential synergistic interaction between NaCl and fludioxonil to hopefully take advantage of the increased osmotic sensitivity for practical

applications. The isolates were grown on PDA amended with NaCl at 0.03, 0.1, 0.3, or 1% plus fludioxonil at 0.05 µg/ml, in comparison with fludioxonil at 0.05 µg/ml only. No synergy was observed (data not shown). *Botrytis* isolates in the eastern U.S. strawberry fields have developed resistance to a wide range of chemical classes of fungicides. Thus far, fludioxonil is one of the few single-site fungicides to which a majority of *B. cinerea* isolates remain sensitive (Hu et al. 2016). *B. fragariae* tended to have a higher level of tolerance to fludioxonil compared with *B. cinerea* (Rupp et al. 2017). Moreover, all of the resistant *B. fragariae* isolates found in this study were able to grow on PDA amended with fludioxonil at 100 µg/ml, except for 21-8-16 (Table 1). Given that *B. cinerea* and *B. fragariae* exist in sympatry (Hu et al. 2018), *B. fragariae* isolates, particularly the resistant isolates, possess the selective advantage. Monitoring of the prevalence of *Botrytis* spp. at different berry growth stages may further understanding of the disease epidemiology.

In conclusion, overexpression of *atrB* was found to be the likely cause of fludioxonil resistance but, uncharacteristically, no variations in *mrr1* were identified in U.S. *B. fragariae* isolates. This suggests that other mechanisms may be involved in regulation of *atrB* expression. A comparative genomic analysis on *B. fragariae* resistant and sensitive isolates may be necessary to uncover the driving forces of *atrB* overexpression in this *Botrytis* sp.

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