Allele-Specific PCR for the Detection of Azoxystrobin Resistance in *Didymella bryoniae*

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Abstract

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Gummy stem blight (GSB), caused by the fungus *Didymella bryoniae*, is considered the most widespread and destructive disease of watermelon in the southeastern United States. The quinone outside-inhibiting (QoI) fungicide azoxystrobin (AZO), which inhibits mitochondrial respiration by binding to the outer, quinone-oxidizing pocket of the cytochrome bc1 (*cyt b*) enzyme complex, was initially very effective in controlling GSB. However, resistance to AZO has been observed in *D. bryoniae* in many watermelon-producing regions. In this study, the DNA sequences of partial *cyt b* genes of four AZO-resistant (AZO-R) and four AZO-sensitive (AZO-S) isolates of *D. bryoniae* confirmed the

amino acid substitution of glycine by alanine at the 143 codon (G143A) in the AZO-R isolates tested. Allele-specific primers were designed to detect the resistant or sensitive allele at codon 143 of the *cyt b* gene, which amplified a 165-bp polymerase chain reaction (PCR) product from genomic DNA of nine AZO-R and nine AZO-S isolates of *D. bryoniae*, respectively. The primer pairs did not amplify DNA from other pathogens tested in the study. The results indicated that the PCR assays developed in the study were specific in differentiating AZO-R and AZO-S isolates and could facilitate AZO resistance detection in *D. bryoniae*.

Gummy stem blight (GSB), caused by the fungus *Didymella bryoniae* (Fuckel) Rhem, is considered the most widespread and destructive disease of watermelon (*Citrullus lanatus* (Thunb.) Matsum. & Nakai) in Georgia and other southeastern U.S. states (9,22). The pathogen infects all aboveground plant parts. Typical symptoms appear as dark-brown, irregularly shaped lesions with a concentric ring pattern that occur near the leaf margins and in the interveinal regions. Lesions may also be found on stems and may expand and girdle the main stem, creating a canker. GSB is primarily controlled using a combination of cultural practices and chemical fungicides. Although cultural practices play an important role in GSB management, they are less efficacious when used alone. When disease pressure is high, fungicides with protective and systemic properties are essential for the successful management of GSB.

The quinone outside inhibitor (QoI) fungicide azoxystrobin (AZO) has been used for control of GSB. QoI fungicides inhibit mitochondrial respiration by binding to the outer, quinone-oxidizing pocket of the cytochrome bc1 (*cyt b*) enzyme complex which, in turn, blocks electron transport and results in energy loss in the fungus (6). With the heavy reliance on systemic fungicides with site-specific modes of action, disease control failures related to fungicide resistance have been noted in several fungal pathogens (3,8,11,15,18,21). In particular, rapid evolution of fungicide-resistant isolates of *D. bryoniae* to AZO was reported within a year of registration of the fungicide for use in commercial watermelon production (12,13,22). Most recently, resistance frequencies of up to 100% have been reported in untreated control plots (23).

The rapid development of resistance to QoI fungicides in fungal plant pathogens has been attributed to a substitution of alanine for glycine at amino acid position 143 (G143A) (6). Substitution of leucine for phenylalanine at amino acid position 129 (F129L) and a substitution of arginine for glycine at amino acid position 137

(G137R) also confer resistance to QoI fungicides in some fungi (4). However, the F129L and G137R mutations have been associated with a lower level of fungicide resistance and do not seriously affect field performance of QoI fungicides (4). In *D. bryoniae*, the rapid development of QoI-fungicide-resistant isolates has been attributed to the G143A mutation (5). However, the sequence of the G143A region of the *cyt b* gene in *D. bryoniae* has not been published.

QoI resistance monitoring can be accomplished using traditional fungicide sensitivity assays or through molecular techniques. Currently, the detection of AZO resistance in *D. bryoniae* is accomplished using conidial germination assays on fungicide-amended medium. Although conidial germination assays are useful in resistance monitoring, these assays are time consuming and labor intensive. Molecular-based detection of resistance would significantly decrease the time and labor required to monitor AZO resistance development. Therefore, the objectives of this study were to sequence the *cyt b* G143A region of *D. bryoniae* isolates, confirm the mutation in the *cyt b* of *D. bryoniae* isolates resistant to AZO, and develop a rapid, allele-specific polymerase chain reaction (PCR) assay to detect AZO resistance.

Materials and Methods

Fungal isolates. Eighteen single-lesion isolates of *D. bryoniae* were used in this study. The isolates were previously collected in 2010 by Thomas et al. (23) from field-grown watermelon plants showing symptoms of GSB in different counties in Georgia (Table 1). Each isolate was previously characterized as AZO-resistant (AZO-R) or AZO-sensitive (AZO-S) using a conidial germination assay with a discriminatory AZO concentration of 10.0 µg/ml and salicylhydroxamic acid (SHAM) at 100 µg/ml (23). Isolates exhibiting greater than 50% relative germination on fungicide-amended medium were considered AZO-R (23). Isolates were grown for 1 to 2 weeks on PDA with small pieces of sterilized filter paper placed on the surface. Colonized filter paper pieces were removed from the plates, air-dried, and then kept at -20°C. Before use, the isolates were assayed again using methods described by Thomas et al. (23) to confirm sensitivity to AZO.

Genomic DNA extraction. *D. bryoniae* isolates were grown in 50 ml of potato dextrose broth at 24°C for 4 days with continuous shaking (100 rpm). Mycelia were harvested (about 100 to 200 mg

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fresh weight), frozen in liquid nitrogen, and then ground into a fine powder using a mortar and pestle. Genomic DNA (gDNA) was extracted from each isolate using a DNeasy Plant Mini kit (Qiagen), following the manufacturer's instructions. DNA concentrations were measured using a NanoDrop 2000 spectrophotometer and adjusted to 50 ng/μl. DNA was stored at -20°C until further use.

Partial sequencing of the gDNA *cyt b* of *D. bryoniae*. Four AZO-R and four AZO-S isolates were used for partial sequencing of *cyt b* in genomic DNA. The primers FG143 and RG143 (Table 2), designed previously by Patel et al. (20) for *Pyrenophora tritici-repentis*, amplified a fragment of the *cyt b* containing the amino acid 143 codon in all eight isolates of *D. bryoniae*. The PCR was performed in a 25-μl volume containing 2.5 μl of 10× PCR buffer, 0.5 μl of 10 mM dNTP, 1.5 μl each of the 10 μM forward and reverse primers, 0.2 μl (1 unit) of *Taq* polymerase, and 2 μl of template DNA (50 ng/μl). All PCR reactions were run in a Bio-Rad T100 Thermal Cycler. The PCR conditions were modified from those reported by Patel et al. (20) as follows: an initial preheat for 5 min at 95°C; followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min;

and a final extension at 72°C for 10 min. PCR products were examined by electrophoresis in a 1% agarose (Fisher Scientific) gel in Tris-borate-EDTA buffer. The gel was amended with 1× GelRed nucleic acid gel stain (Biotium, Inc.) and visualized directly in a Bio-Rad Chemi XRS Gel Documentation system (Bio-Rad). The PCR products were purified using QIAquick PCR Purification kit (Qiagen), according to the manufacturer's instructions. The purified PCR products were sent for direct sequencing in both directions (Eurofins MWG Operon). The sequences obtained from the four AZO-R and four AZO-S isolates were compared using the Clustal Omega program (<http://www.ebi.ac.uk/Tools/msa/clustalo/>).

Development of *D. bryoniae* allele-specific PCR primers for detection of AZO resistance. The primer sets DB-F/DB-RR and DB-F/DB-RS (Table 2) were designed and used to amplify the *cyt b* sequences with and without the G143A mutation, respectively, by matching the desired allele and mismatching the other allele at the 3' end of the primer. The reverse primers DB-RR and DB-RS were designed so that they contained a C or G nucleotide, respectively, at the first position at the 3' end. A third primer set, DB-FC/DB-RC (Table 2), was designed to amplify both alleles.

To confirm species specificity of the primer sets DB-F/DB-RR, DB-F/DB-RS, and DB-FC/DB-RC, nine AZO-R and nine AZO-S *D. bryoniae* isolates and other fungal species isolated from watermelon, including *Pythium ultimum*, *Phytophthora capsici*, *Fusarium oxysporum* f. sp. *niveum*, *Sclerotium rolfsii*, *Rhizoctonia solani*, and *Pseudoperonospora cubensis*, were tested. Genomic DNA was extracted from all species as described above.

All PCR amplifications were performed in a 25-μl volume containing 2.5 μl of 10× PCR buffer, 0.5 μl of 10 mM dNTP, 2 μl each of the 10 μM forward and reverse primers, 0.2 μl (1 unit) of *Taq* polymerase, and 2 μl of template DNA (50 ng/μl). The PCR conditions were as follows: an initial preheat for 3 min at 95°C; followed by 25 cycles of denaturation at 95°C for 10 s, annealing at 60°C for 10 s, and extension at 72°C for 15 s; and a final extension at 72°C for 3 min. PCR products were examined by electrophoresis, as described earlier.

Results

Analysis of partial *cyt b* sequence of *D. bryoniae* gDNA. The primers FG143 and RG143 amplified a single approximately 265-bp DNA fragment in the four AZO-R isolates and four AZO-S isolates of *D. bryoniae* tested. Sequence analysis revealed the presence of a single nucleotide substitution of cytosine for guanine (GGT to GCT) at the amino acid codon 143 in all AZO-R isolates (Fig. 1). This nucleotide substitution was not present in any of the AZO-S isolates tested (Fig. 1).

Genomic DNA allele-specific PCR. The AZO-R allele-specific primer pair DB-F/DB-RR consistently amplified a 165-bp PCR

Table 1. Source of *Didymella bryoniae* isolates used in this study^a

Isolate	Georgia county	AZO sensitivity ^b
C2-8	Tift	S
R204-1-1	Tattnall	S
UR3-5	Tattnall	S
R501-3-1	Tattnall	S
R501-3-2	Tattnall	S
R501-3-4	Tattnall	S
R501-3-5	Tattnall	S
R606-3-1	Tattnall	S
R606-3-4	Tattnall	S
C2-1	Tift	R
B105-3-3	Tift	R
C5-1	Tift	R
C2-5	Tift	R
C2-7	Tift	R
C5-7	Tift	R
C5-8	Tift	R
UR3-1	Tattnall	R
UR5-9	Tattnall	R

^a Isolates were collected in 2010 from watermelon fields by Thomas et al. (23).

^b Previously determined using in vitro fungicide sensitivity assay by Thomas et al. (23). Isolates exhibiting >50% relative germination (RG) on azoxystrobin (AZO)-amended medium at a discriminatory concentration of 10.0 μg/ml were considered resistant (R). Isolates exhibiting <50% RG were considered sensitive (S).

Table 2. Oligonucleotide primers used in this study

Primer name	Sequence	Feature ^a	Reference
FG143	5' GCA GCT TTA GCC CTT GGT AA 3'	Partial <i>cyt b</i> covering codon 143	Patel et al. (20)
RG143	5' CTG CGC TAT TTT TAA TAT AGG TTC CTG 3'	Partial <i>cyt b</i> covering codon 143	Patel et al. (20)
DB-F	5' CGT ATA GTC TCT GAG GAA CC 3'	Forward primer for AZO-R and AZO-S	This study
DB-RR	5' GCA GAT GTC ATT ATG AGG 3'	AZO-R allele specific reverse primer	This study
DB-RS	5' GCA GAT GTC ATT ATG AGC 3'	AZO-S allele specific reverse primer	This study
DB-FC	5' CAG ATG TCA TTA TGA GGT GCT ACA 3'	Forward primer for both alleles	This study
DB-RC	5' CCG CTT CAC AAA GGC CTA TAA 3'	Reverse primer for both alleles	This study

^a Abbreviations: *cyt b* = cytochrome b gene, AZO-R = azoxystrobin-resistant, and AZO-S = azoxystrobin-sensitive.

gDNA_AZO-R	TTTGATAACAAGATTAAATACTACTTTTTTACAATAAGTAAGTTTGTATGTGCTGCCCTA
gDNA_AZO-S	TTTGATAACAAGATTAAATACTACTTTTTTACAATAAGTAAGTTTGTATGTGCTGCCCTA
gDNA_AZO-R	CGGGCAGATGTCATTATGAGCTGCTACAGTTAAAAAGGCGAAGTAATAGCTGTAGTAAAA
gDNA_AZO-S	CGGGCAGATGTCATTATGAGGTGCTACAGTTAAAAAGGCGAAGTAATAGCTGTAGTAAAA

Fig. 1. Cytochrome b sequences from genomic DNA (gDNA) of azoxystrobin-resistant (AZO-R) and -sensitive (AZO-S) isolates of *Didymella bryoniae*. Highlighted letters indicate the nucleotide change at amino acid position 143 that confers resistance to AZO.

product from nine AZO-R isolates of *D. bryoniae* tested but not from any of the nine AZO-S isolates (Fig. 2). The AZO-S allele-specific primer pair DB-F/DB-RS consistently amplified a 165-bp PCR product from nine AZO-S isolates of *D. bryoniae* but not from any of the nine AZO-R isolates tested (Fig. 3). Both alleles were consistently amplified with primer set DB-FC/DB-RC (Fig. 4). DNA of *Pythium ultimum*, *Phytophthora capsici*, *F. oxysporum* f. sp. *niveum*, *S. rolfii*, *R. solani*, or *Pseudoperonospora cubensis* were not amplified by primer pairs DB-FC/DB-RC (Fig. 4) and DB-F/DB-RR or DB-F/DB-RS (*data not shown*).

Discussion

In this study, a fragment of the *cyt b* gene containing the G143A site was amplified in genomic DNA of *D. bryoniae*. The highly conserved nature of the *cyt b* region containing the G143A site

(7,20) allowed us to use previously published primer sets for closely related ascomycetes to screen the *cyt b* gene of *D. bryoniae*. Prior to this study, there were no published *cyt b* sequences for *D. bryoniae*. The G143A mutation was detected in all AZO-R isolates tested but not in AZO-S isolates. These results are in agreement with a previous report that the G143A mutation is present in and responsible for resistance to QoI fungicides in *D. bryoniae* (5). These findings also further validate the results obtained from the conidial germination assays used to characterize isolates as either AZO-R or AZO-S, because the G143A mutation was present in all AZO-R phenotypes and absent in all AZO-S phenotypes.

Molecular methods to differentially detect *D. bryoniae* resistant to QoI fungicides have not been reported, though several qualitative and quantitative molecular techniques have been used to iden-

Fig. 2. Allele-specific polymerase chain reaction using genomic DNA with primer pair DB-F/DB-RR for the detection of azoxystrobin-resistant (AZO-R) isolates of *Didymella bryoniae*. AZO-sensitive isolates: S1 = UR3-5, S2 = R501-3-1, S3 = C2-8, S4 = R204-1-1, S5 = R501-3-2, S6 = R501-3-4, S7 = R501-3-5, S8 = R606-3-1, S9 = R606-3-4. AZO-R isolates: R1 = C2-1, R2 = C5-1, R3 = B105-3-3, R4 = UR5-9, R5 = C2-5, R6 = C2-7, R7 = C5-7, R8 = C5-8, R9 = UR3-1. C = negative control, M = 1-kb DNA ladder (Qiagen).

Fig. 3. Allele-specific polymerase chain reaction using genomic DNA with primer pair DB-F/DB-RS for the detection of azoxystrobin-sensitive (AZO-S) isolates of *Didymella bryoniae*. AZO-S isolates: S1 = UR3-5, S2 = R501-3-1, S3 = C2-8, S4 = R204-1-1, S5 = R501-3-2, S6 = R501-3-4, S7 = R501-3-5, S8 = R606-3-1, S9 = R606-3-4. AZO-resistant isolates: R1 = C2-1, R2 = C5-1, R3 = B105-3-3, R4 = UR5-9, R5 = C2-5, R6 = C2-7, R7 = C5-7, R8 = C5-8, R9 = UR3-1. C = negative control, M = 1-kb DNA ladder (Qiagen).

Fig. 4. Allele-specific polymerase chain reaction using genomic DNA with primer pair DB-FC/DB-RC for the detection of azoxystrobin-sensitive (AZO-S) and -resistant (AZO-R) isolates of *Didymella bryoniae*. AZO-S isolates: S1 = UR3-5, S2 = R501-3-2, S3 = C2-8, S4 = R204-1-1. AZO-R isolates: R1 = C2-1, R2 = C5-1, R3 = B105-3-3, R4 = UR5-9. Other species: Pc = *Phytophthora capsici*, Pu = *Pythium ultimum*, Rs = *Rhizoctonia solani*, Sr = *Sclerotium rolfii*, Dm = *Pseudoperonospora cubensis*, Fu = *Fusarium oxysporum* f. sp. *niveum*. Neg = negative control, M = 100 bp DNA ladder (Qiagen).

tify resistance to QoI fungicides in a number of other plant pathogenic fungi (1–3,8,14,16,17,24). Of those techniques, allele-specific PCR has been used for both qualitative and quantitative detection of QoI resistance in *Blumeria graminis* f. sp. *tritici*, *Alternaria* spp., *Venturia inaequalis*, and *Botrytis cinerea* (2,3,8,16,17). In the present study, allele-specific PCR was used to detect AZO resistance in isolates of *D. bryoniae*. In addition to primers designed to amplify the AZO-R and AZO-S alleles, a third primer set was designed that amplified both allele types as a control. All allele-specific primer sets developed in this study were determined to be specific to *D. bryoniae*, because they did not amplify any other fungal species tested.

To our knowledge, this is the first report in which molecular-based detection has been developed to identify resistance to AZO in *D. bryoniae*. Traditionally, conidial germination assays have been employed for AZO resistance detection in *D. bryoniae* (10,19,22,23). Although conidial germination assays are useful in AZO resistance detection, these assays can be time consuming and laborious. The allele-specific PCR assays significantly decrease time and labor required to detect AZO resistance in *D. bryoniae*. The results of this study also provide the basis for development of a quantitative molecular assay that will be useful in studying epidemiological aspects of GSB. However, further research is needed in order to confirm that the allele-specific PCR protocols developed in this study can be used to successfully detect AZO resistance in GSB-infected plant samples.

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