Differences in Sensitivity to a Triazole Fungicide Among *Stagonosporopsis* Species Causing Gummy Stem Blight of Cucurbits

Hao-Xi Li, Department of Plant Pathology, University of Georgia, Athens, GA 30602; Katherine L. Stevenson, Department of Plant Pathology, University of Georgia, Tifton, GA 31793; and Marin T. Brewer, Department of Plant Pathology, University of Georgia, Athens, GA 30602

Abstract

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Gummy stem blight (GSB) is a destructive disease of cucurbits caused by three closely related *Stagonosporopsis* species. In the southeastern United States, GSB management relies heavily on triazole fungicides. Our objectives were to determine if resistance to triazoles has developed in populations of GSB fungi in the southeastern United States, and if so, to investigate the molecular basis of resistance. A tebuconazole sensitivity assay was conducted on 303 *Stagonosporopsis citrulli* and 19 *S. caricae* isolates collected from the southeastern United States in 2013 and 2014, as well as three *S. citrulli*, three *S. cucurbitacearum*, and six *S. caricae* isolates from other regions or years. Tebuconazole resistance was detected for all

19 *S. caricae* isolates from the southeastern United States and one *S. caricae* isolate from Brazil. All *S. citrulli* and *S. cucurbitacearum* isolates were sensitive to tebuconazole. For resistant and sensitive isolates of *S. caricae*, coding and promoter regions of the target gene *Cyp51* were sequenced and expression levels of *Cyp51* and *ScAtrG* (an ATP-binding cassette transporter) were measured. Tebuconazole resistance was not associated with mutations within *Cyp51*, multiple copies of *Cyp51*, changes in the promoter region, or increased expression of *Cyp51* or *ScAtrG*. Tebuconazole resistance may explain the increase in frequency of *S. caricae* isolates recovered from GSB-infected cucurbits in Georgia.

Cucurbits are susceptible to gummy stem blight (GSB), a destructive foliar disease that has caused severe epidemics in the southeastern United States over the past several decades (Gusmini et al. 2005; Keinath et al. 1995; Schenck 1968; St. Amand and Wehner 1995). These epidemics can have major economic impacts, because more than one third of the cucurbits produced in the United States are from southeastern states, including Georgia, Florida, and South Carolina, which, alone, contribute approximately \$356 million to the local economy (NASS 2015). GSB not only decreases yield but, also, reduces fruit quality (Keinath 2000). GSB is caused by three morphologically similar but genetically distinct *Stagonosporopsis* species, *S. cucurbitacearum* (syn. *Didymella bryoniae*), *S. citrulli*, and *S. caricae* (Stewart et al. 2015). All three fungal species are necrotrophs with wide host ranges among cucurbits (Chiu and Walker 1949; Keinath 2011; Stewart et al. 2015) affecting aboveground, vegetative structures at any growth stage after seed germination (Wiant 1945). The three *Stagonosporopsis* species are pathogenic to cucurbits with similar virulence; however, *S. caricae* is also pathogenic to papaya (Stewart et al. 2015), causing fruit and stem rot and leaf spot of papaya. Until recently, only *S. citrulli* was recovered from GSB-infected cucurbits in the southeastern United States (Stewart et al. 2015). In 2013, four of 246 GSB fungi sampled from four watermelon fields in the southeastern United States were identified as *S. caricae* (Li and Brewer 2016). The four *S. caricae* isolates came from a single field in Tift County, Georgia. In 2014, 15 of 76 GSB fungi sampled from a single watermelon field in Cook County, Georgia were identified as *S. caricae* (Li and Brewer 2016).

Cultivars of watermelon (*Citrullus lanatus*), cucumber (*Cucumis sativus*), or muskmelon (*Cucumis melo*) resistant to *Stagonosporopsis* species are not currently available (Keinath 2014), so management of GSB requires both cultural and chemical control strategies. It is recommended that growers use only certified pathogen-free seed and seedlings (Sitterly and Keinath 1996). To limit local survival of

GSB fungi on debris, a minimum 2-year rotation with noncucurbit crops is recommended (Keinath 1996, 2000). Other cultural practices include incorporation of infested residue to reduce survival on plant debris (Sitterly and Keinath 1996), avoidance of overhead irrigation to reduce periods of leaf wetness (Thomas et al. 2012b), and soil fumigation with cabbage residue amendments (Keinath 1996). Nonetheless, fungicide application is by far the most effective option for GSB management (Keinath 2000, 2012; Thomas 2012b). Unfortunately, GSB fungi in the southeastern United States have repeatedly and rapidly evolved resistance to several classes of fungicides. In the mid-1990s, thiophanate-methyl, a methyl benzimidazole carbamate (MBC) fungicide (Fungicide Resistance Action Committee, FRAC #1) (FRAC 2016), was deemed ineffective against GSB due to the development of resistance in pathogen populations (Keinath and Zitter 1998). Initially, azoxystrobin, a quinone outside inhibitor (QoI) fungicide (FRAC #11), provided excellent control of GSB, but by the end of 1990s, cases of resistance appeared in Georgia (Stevenson et al. 2004) and South Carolina (Keinath 2009). Boscalid, a succinate dehydrogenase inhibitor (SDHI) fungicide (FRAC #7), provided control for GSB until 2007 (Seebold and Langston 2003), when widespread resistance developed in Georgia (Thomas et al. 2012a) and South Carolina (Keinath 2012). GSB fungi remain sensitive to triazoles, the largest subgroup of demethylation inhibitors (DMI) (FRAC #3) (Keinath and Hansen 2013; Thomas et al. 2012b). DMI fungicides inhibit ergosterol biosynthesis by targeting lanosterol 14 α -demethylase, which is encoded by the gene *Cyp51* (Kuck et al. 2002). Triazoles are used against a broad spectrum of fungi in medical and agricultural settings (Moore et al. 2000; Price et al. 2015; van den Bossche et al. 2003).

In Georgia (Langston 2014), Florida (Dufault and Paret 2014), and South Carolina (Keinath and Miller 2014), management of GSB relies on triazoles, which is concerning, because resistance to triazoles has been observed for many other plant-pathogenic fungi (Cools et al. 2013; Price et al. 2015). However, unlike the qualitative resistance observed for single-site fungicides such as azoxystrobin, resistance to triazoles is quantitative, often resulting in development of a continuum of resistance phenotypes (Cools and Fraaije 2013). Mechanisms of resistance to triazoles include: (i) single-site mutations in the coding region of *Cyp51* leading to changes in the amino acid sequence of the protein, making it unrecognizable by the fungicide (Cools et al. 2011; Frenkel et al. 2015; Schmitz et al. 2014), (ii)

Corresponding Author: M. T. Brewer; E-mail address: mtbrewer@uga.edu

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overexpression of *Cyp51* due to changes in the promoter region (Cools et al. 2012; Schnabel and Jones 2001) or other unknown enhancers of transcription (Bolton et al. 2012; Ma and Tredway 2013), (iii) multiple copies of *Cyp51* genes (Brunner et al. 2016), and (iv) overexpression of transporters that control the efflux of fungicides (Kretschmer et al. 2009). Common transporter groups include ATP-binding cassette (ABC) transporters and major facilitator superfamily (MFS) transporters (Cools et al. 2013). The objectives of this study were to determine if resistance to triazole fungicides has developed in field populations of GSB fungi in the southeastern United States, and, if so, to investigate the molecular basis of triazole resistance in GSB fungi.

Materials and Methods

Isolates of GSB fungi. A total of 322 fungal isolates was collected from five watermelon (*Citrullus lanatus*) fields in Georgia, Florida, and South Carolina in July 2013 and 2014 (Table 1). There were 53 isolates sampled from a field in Cook County, Georgia in 2013; 76 isolates sampled from Cook County, Georgia in 2014; 99 isolates sampled from Tift County, Georgia in 2013; 72 isolates sampled from Suwannee County, Florida in 2013; and 22 isolates sampled from Colleton County, South Carolina in 2013 (Table 1). The detailed sampling methods and isolation procedures for these fungi have been described previously (Li and Brewer 2016). Nineteen of the field isolates were previously identified as *S. caricae*, while the remaining 303 isolates were identified as *S. citrulli* (Brewer et al. 2015; Li and Brewer 2016). In addition, twelve GSB or papaya fruit rot isolates, including three *S. citrulli*, three *S. cucurbitacearum*, and six *S. caricae* isolates, were screened for resistance (Table 1). These isolates were collected from different cucurbit-growing regions across the United States, from South America, and from additional hosts, including cucumber (*Cucumis sativus*), muskmelon (*Cucumis melo*), butternut squash (*Cucurbita moschata*), and papaya (*Carica papaya*) (Stewart et al. 2015). All isolates were recovered on quarter-strength potato dextrose agar (qPDA) from filter paper maintained at -20°C . In total, 306 *S. citrulli* isolates, three *S. cucurbitacearum* isolates, and 25 *S. caricae* isolates were tested for tebuconazole sensitivity.

Fungicide sensitivity assay. Tebuconazole was selected to determine triazole sensitivity because it is widely used for GSB management in the southeastern United States. We used an in vitro mycelial growth assay developed by Thomas et al. (2012a) that compares mycelial growth on a tebuconazole-amended medium ($3.0\text{ }\mu\text{g/ml}$) with mycelial growth on a control medium. Technical grade tebuconazole (97.5% active ingredient; Bayer CropScience, Kansas City, MO) was dissolved in acetone to obtain a stock solution of 30 mg/ml . A $10\times$ dilution of the stock solution was prepared in 1 ml of acetone and was added to 1 liter of autoclaved, cooled PDA to obtain a concentration of $3.0\text{ }\mu\text{g}$ of tebuconazole per milliliter. A control medium was prepared by adjusting PDA to a final acetone concentration of 0.1% by volume.

For each isolate, 5-mm diameter mycelial plugs were obtained from the edge of a 1-week-old colony, using a cork borer, and were placed in petri dishes of tebuconazole-amended and control media. Isolates were incubated in the dark at 25°C for 4 days. Mycelial growth was estimated as the average diameter of the colony less 5 mm for the mycelial plug. The relative growth (RG) for each isolate was calculated as the ratio of mycelial growth on the tebuconazole-amended medium to the mycelial growth on the control medium. Based on the previously developed assay (Thomas et al. 2012a), a phenotype of resistant to tebuconazole was assigned to GSB isolates with RG values greater than 25% and sensitive to tebuconazole was assigned to isolates with RG values less than 25%. We observed that resistant isolates could be further divided into two phenotypic categories, which consisted of highly resistant isolates with RG values greater than 75% and moderately resistant isolates with RG from 25 to 75%. The mycelial growth assay was repeated for isolates with highly resistant and moderately resistant phenotypes.

Identification of *Cyp51* in draft genomes of *Stagonosporopsis* species. Draft genomes of nine GSB or papaya fruit rot isolates including three *S. citrulli*, three *S. cucurbitacearum*, and three *S. caricae* were assembled. A high quality DNA extraction method (Fulton et al. 1995) was used. Cetyl trimethylammonium bromide (CTAB) lysis buffer was prepared by mixing 6.5 ml of buffer A (0.35 M sorbitol, 0.1 M Tris-HCl, pH 9, and 5 mM EDTA, pH 8), 6.5 ml of buffer B (0.2 M Tris-HCl, pH 9, 50 mM EDTA, pH 8, 2 M NaCl, 2% CTAB),

Table 1. Resistance or sensitivity to tebuconazole for isolates of *Stagonosporopsis* spp. causing gummy stem blight of cucurbits or fruit rot of papaya

Species (isolate)	Sampling location (year ^a)	Original host	No. of isolates	Tebuconazole phenotype ^b
Field isolates				
<i>S. citrulli</i>	Cook Co., Georgia (2013)	Watermelon (<i>Citrullus lanatus</i>)	53	S
<i>S. citrulli</i>	Tift Co., Georgia (2013)	Watermelon	95	S
<i>S. citrulli</i>	Suwannee Co., Florida (2013)	Watermelon	72	S
<i>S. citrulli</i>	Colleton Co., South Carolina (2013)	Watermelon	22	S
<i>S. citrulli</i>	Cook Co., Georgia (2014)	Watermelon	61	S
<i>S. caricae</i>	Tift Co., Georgia (2013)	Watermelon	2	HR
<i>S. caricae</i>	Tift Co., Georgia (2013)	Watermelon	2	MR
<i>S. caricae</i>	Cook Co., Georgia (2014)	Watermelon	14	HR
<i>S. caricae</i>	Cook Co., Georgia (2014)	Watermelon	1	MR
Isolates with sequenced genomes				
<i>S. citrulli</i> (C5-5)	Tift Co., Georgia (2010)	Watermelon	1	S
<i>S. citrulli</i> (DIDYNY)	Onondaga Co., New York (N.A.)	Muskmelon (<i>Cucumis melo</i>)	1	S
<i>S. citrulli</i> (AcSq5)	Johnson Co., North Carolina (2000)	Acorn squash (<i>Cucurbita pepo</i>)	1	S
<i>S. cucurbitacearum</i> (RT2)	Michigan (1997)	Butternut squash (<i>Cucurbita moschata</i>)	1	S
<i>S. cucurbitacearum</i> (GSB26)	Tompkins Co., New York (1992)	Muskmelon	1	S
<i>S. cucurbitacearum</i> (GSB29)	New York (1992)	Watermelon	1	S
<i>S. caricae</i> (RG3)	California (N.A.)	Watermelon	1	S
<i>S. caricae</i> (ATCC5257)	Hawaii (N.A.)	Papaya (<i>Carica papaya</i>)	1	S
<i>S. caricae</i> (C166)	Michigan (N.A.)	Cucumber (<i>Cucumis sativus</i>)	1	S
Other <i>S. caricae</i> isolates				
<i>S. caricae</i> (2245)	Brazil (N.A.)	Unknown cucurbit	1	HR
<i>S. caricae</i> (CBS102399)	Brazil (N.A.)	Papaya	1	S
<i>S. caricae</i> (CBS248.90)	Chile (N.A.)	Papaya	1	S

^a N.A. indicates that the sampling year was unknown.

^b S indicates sensitive (relative growth (RG) < 25%), MR indicates moderately resistant (25% < RG < 75%), and HR indicates highly resistant (RG > 75%), based on in vitro mycelial growth assay on tebuconazole-amended media.

2.6 ml of buffer C (5% sarkosyl), 1.75 ml of polyvinylpyrrolidone (0.1%), and 1.25 μ l proteinase K. Approximately 500 mg of mycelium growing at 25°C in the dark for 7 days on qPDA covered with sterile cellophane was removed and added to CTAB lysis buffer. The mixture was agitated with two 5-mm glass beads (VWR Soda Lime, Radnor, PA) at 1,750 rpm for 2 min in a 2010 Geno/Grinder (SPEX SamplePrep, Metuchen, NJ). The mixture was then inverted 10 times after adding 5.75 ml of potassium acetate (5 M), and was incubated on ice for 30 min, followed by centrifugation for 20 min at 14,000 $\times$ g. The supernatant was added to one volume of chloroform-isoamylalcohol (vol/vol 24:1), followed by centrifugation for 10 min at 14,000 $\times$ g. The supernatant was added to 100 μ l of RNase A (10 mg/ml) and was incubated at 37°C for 120 min. An equal volume of isopropanol and $1/10$ volume of sodium acetate was added; the mixture was then incubated at 25°C for 5 min, followed by centrifugation at 14,000 $\times$ g for 30 min. The supernatant was discarded and the pellet was dissolved in 100 μ l of deionized H₂O, after being rinsed twice with 70% ethanol and air-dried.

Genomic DNA for each isolate was submitted to the Georgia Genomics Facility (Athens, GA) for next-generation sequencing library preparation, followed by sequencing reactions using the Illumina MiSeq platform according to the PE300 protocol (Illumina Inc., San Diego, CA). Files of the forward and reverse reads of each isolate with optimal *k*-mer values of 64 (substrings of length *k* in DNA sequence data) were assembled de novo into draft genomes, using ABySS v.1.3.6 (Simpson et al. 2009) on the Georgia Advanced Computing Resource Center Linux cluster.

Nucleotide sequence of the *Cyp51* gene from the annotated genome of *Didymella exigua* (scaffold 10: 784720-786692) (Nordberg et al. 2014) and paralogs in the genomes of *Gibberella zeae* (GenBank accession numbers FJ216393 and FJ216400) (Yin et al. 2009) and *Aspergillus fumigatus* (GenBank accession numbers AF338659 and AF338660) (Mellado et al. 2001) were used to search for homologs in each draft genome of the nine GSB isolates, using megaBLAST (Altschul et al. 1990). With pairwise megaBLAST, the *Cyp51* regions identified in GSB isolates and from the *D. exigua* genome were compared with paralogs from *G. zeae* and *A. fumigatus*.

Sequencing of *Cyp51* from *Stagonosporopsis caricae*. The *Cyp51* genes from 19 isolates of *S. caricae* were aligned and compared. In addition to the sequences identified in the three draft genomes of tebuconazole-sensitive *S. caricae* isolates (Table 1), 16 isolates were polymerase chain reaction (PCR)-amplified and sequenced including: 11 isolates highly resistant to tebuconazole (GA6057 and GA6081 from Tift County, Georgia, sampled in 2013; GA8002, GA8007, GA8016, GA8036, GA8051, GA8061,

GA8091, and GA8098 from Cook County, Georgia, sampled in 2014; and 2245 from Brazil), three isolates moderately resistant to tebuconazole (GA6011 and GA6074 from Tift County, Georgia, sampled in 2013; GA8025 from Cook County, Georgia, sampled in 2014), and two isolates sensitive to tebuconazole (CBS102399 from Brazil and CBS248.90 from Chile). Genomic DNA was extracted from fresh mycelium, using a method modified from Lee et al. (1988). Approximately 100 mg of mycelium from each isolate was harvested, after incubation at 25°C in the dark for 4 days, by removing the mycelium from qPDA covered with sterile cellophane. The mycelium was mixed with 1 ml of mini-prep lysis buffer (50 mM EDTA, pH 8, 100 mM Tris, pH 8, 3.5% sodium dodecyl sulfate, 500 μ g of proteinase K per milliliter, and 1% sodium bisulfite) by vortexing for 1 min. The mixture was then incubated for 15 min at 65°C, followed by centrifugation for 5 min at 14,000 $\times$ g. The supernatant was added to 200 μ l of ammonium acetate (7.5 M), and was vortexed for 10 s. The mixture was placed on ice for 15 min, and then, was centrifuged at 14,000 $\times$ g for 3 min. The supernatant was precipitated with an equal volume of isopropanol and was centrifuged at 14,000 $\times$ g for 5 min. The pellet was then resuspended in 100 μ l of sterilized deionized H₂O, after rinsing twice with 70% ethanol and air-drying. The DNA was stored at -20°C.

Based on the *Cyp51* sequence from the draft genome of *S. caricae*, two pairs of primers were designed, using Primer3 (Rozen and Skaletsky 2000), to amplify the promoter and coding regions of *Cyp51* (Table 2). PCR was conducted using a 20- μ l volume reaction, prepared with reagents from TaKaRa Bio Inc. (Otsu, Japan): 3.5 μ l of 10 $\times$ buffer, 3.0 μ l of dNTP mixture (2.5 mM), 5 μ l of TaKaRa ExTaq (5U/ μ l), 1.5 μ l of each pair of primers (10 μ M), and 1 μ l of template DNA (3 to 20 ng). The thermal cycling conditions were initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 57°C for 30 s, and extension at 72°C for 2 min, with a final extension step at 72°C for 10 min. PCR products were prepared for sequencing using USB ExoSAP-IT (Affymetrix, Inc., Cleveland, OH) according to the manufacturer's protocol. To cover the entire promoter and coding regions of *Cyp51*, six primers were designed for the sequencing reactions (Table 2). Sequencing reactions for each isolate were prepared by mixing 1 μ l of prepared PCR products and 1 μ l of each sequencing primer (3.3 μ M). Products were submitted to the Georgia Genomics Facility, where sequencing reactions were conducted using Big Dye terminator chemistry and Ampli-Taq-FS DNA polymerase, followed by electrophoresis on a 3730xl 96-capillary DNA analyzer (Applied Biosystems, Foster City, CA). The sequences of the *Cyp51* genes and upstream regions were aligned using Geneious 7.0.6 (Biomatters

Table 2. Nucleotide sequence and purpose of primers used in this study

Primer	Sequence	Purpose
ScCyp51_F-175	5'-TTTATCGCCCCGATCTGCTCC-3'	Amplification of <i>Cyp51</i> coding region
ScCyp51_R1774	5'-TAACACACCAGGTAGTGCGG-3'	Sequencing of <i>Cyp51</i> coding region
ScCyp51u_F-1314	5'-TAACCACCGTCGCTACACAC-3'	Amplification of <i>Cyp51</i> coding region
ScCyp51u_R215	5'-GGGTCCATGCCATAGGTGAC-3'	Sequencing of <i>Cyp51</i> coding region
ScCyp51_F289	5'-GAGGCAACGCGTGACTC-3'	Amplification of <i>Cyp51</i> promoter region
ScCyp51_F590	5'-GCACTCCGCTCCTACGTTAC-3'	Sequencing of <i>Cyp51</i> promoter region
ScCyp51_R1055	5'-CCAGGCGAAGGAGAATCCAA-3'	Amplification of <i>Cyp51</i> coding region
ScCyp51_R1390	5'-AGCTTCTGTTCTTCTCTTTGT-3'	Sequencing of <i>Cyp51</i> coding region
ScCyp51u_F-839	5'-GGTGCCACGAAGATGCTGAA-3'	Sequencing of <i>Cyp51</i> coding region
ScCyp51u_R-188	5'-GCGATAAACTGACGACACGC-3'	Sequencing of <i>Cyp51</i> promoter region
ScCyp51_F1212	5'-CACGCACCCATTCACTCCATT-3'	Sequencing of <i>Cyp51</i> promoter region
ScCyp51_R1321	5'-GAGAAGCCAGGCGAAGACATC-3'	Determining expression level of <i>Cyp51</i>
ScAct_F	5'-CTCCCATCAACCCCAAGTCCA-3'	Determining expression level of <i>Actin</i>
ScAct_R	5'-CAGCCTGGATGGAGACGTAGA-3'	Determining expression level of <i>Actin</i>
ScAtrG_F	5'-ACTGCTACTCCTAGCCAAGGGT-3'	Determining expression level of <i>AtrG</i>
ScAtrG_R	5'-GTCTGACCGTGTTCACCGATG-3'	Determining expression level of <i>AtrG</i>

Ltd., Auckland, New Zealand). The sequences from resistant isolates and moderately resistant isolates were compared with sequences from sensitive isolates to identify polymorphic sites responsible that were associated with tebuconazole resistance. Polymorphic sites in the coding regions were identified as synonymous (same amino acid) or nonsynonymous (different amino acid).

RNA extraction and reverse transcription. Twelve isolates were selected to measure expression levels of *Cyp51* and *ScAtrG* (an ABC transporter gene). These isolates included four *S. caricae* isolates highly resistant to tebuconazole (GA8007 and GA8016 from Cook County, Georgia, sampled in 2014, and 2245, sampled from Brazil) or moderately resistant to tebuconazole (GA6074 from Tift County Georgia, sampled in 2013), and four *S. caricae* isolates sensitive to tebuconazole (C166, RG3, ATCC5257, and CBS102399). For comparison among GSB species, expression levels were measured in two sensitive *S. citrulli* isolates (GA8073 from Cook County, Georgia, sampled in 2014, and C5-5) and two sensitive *S. cucurbitacearum* isolates (GSB26 and RT2).

Approximately 100 mg of fresh mycelium of each isolate was harvested from PDA amended with tebuconazole (0.3 µg/ml), after incubating at 25°C in the dark for 7 days, by removing the mycelium from PDA covered with sterile cellophane. Mycelium was added to PureLink lysis buffer (Life Technologies, Carlsbad, CA) containing 1% 2-mercaptoethanol. Two 5-mm glass beads were added to each tube, and the mixtures were homogenized at 1,750 rpm for 2 min, using a 2010 Geno/Grinder. The manufacturer's instructions for the PureLink RNA mini kit were followed for extraction of total RNA from each isolate. The RNA quality was determined by electrophoresis on a 1% (wt/vol) agarose gel and the concentration was measured, using a Spectrophotometer ND-1000 (NanoDrop Technologies, Inc., Wilmington, DE).

Reverse transcription was carried out with a PrimerScript RT reagent kit (TaKaRa Bio Inc.). The genomic DNA elimination reaction required a 15-µl volume mixture containing 3.5 µl of RNA extract (1–2 ng), 3 µl of 5× gDNA eraser buffer, and 1.5 µl of gDNA eraser. The mixture was incubated at 42°C for 2 min. Reverse transcription was conducted in a 30-µl volume containing 15 µl of gDNA elimination reaction solution from the previous step, 4.5 µl of 5× PrimeScript buffer 2, 1.5 µl of PrimeScript RT enzyme mix I, and 1.5 µl of RT primer mix. The mixture was incubated at 37°C for 15 min, followed by 85°C for 2 s. The complementary DNA (cDNA) was stored at –20°C. To replicate the experiment, the same 12 isolates were grown a second time, then, the RNA was extracted and reverse transcribed to cDNA as described above.

Expression level of *Cyp51* and *ScAtrG* by real-time PCR. A pair of primers (Table 2) with 100% similarity to all three species was developed for amplification of a 110-bp fragment of *Cyp51* for real-time PCR. *Actin*, a gene with a constant expression level, was used as an internal control in the real-time PCR. *Actin* was identified in the draft genomes of the nine GSB fungi by MegaBLAST with the homolog from *Didymella rabiei* (GenBank accession KM244530) (Kim et al. 2015). Primers (Table 2) with 100% similarity to all three GSB species were designed to amplify a 93-bp fragment of *Actin*.

To investigate if increased fungicide efflux from the cell by a transporter was associated with tebuconazole resistance in *S. caricae*, the expression levels of a transporter gene were measured by real-time PCR. Using homologs of transporter genes in *Botrytis fuckeliana*, the draft genomes of nine GSB isolates were searched by megaBLAST. The genes included two ABC transporters, *BcatrB* (GenBank accession AJ006217) (Schoonbeek et al. 2001) in the pleiotropic drug resistance (ABC-G) group and *BcatrD* (GenBank accession AJ272521) (Hayashi et al. 2001) in the multidrug resistance (ABC-B) group, and one type of MFS transporter, *MfsM2* (GenBank accession CQ292707) (Kretschmer et al. 2009). Only a homolog of the pleiotropic drug resistance group (ABC-G), which we named *ScAtrG*, was identified in the draft genomes of GSB fungi. A set of primers (Table 2) with 100% similarity to the three *Stagonosporopsis* species was designed for the amplification of a 66-bp fragment of *ScAtrG* by real-time PCR.

Real-time PCR reactions were conducted to detect the expression levels of *Cyp51*, *ScAtrG*, and *Actin* on 12 GSB isolates, using the StepOnePlus Real-Time PCR system (Life Technologies). Three

technical replicate reactions were conducted for each gene for each of the 12 isolates. Each reaction was conducted in 20-µl volumes containing 2.0 µl of cDNA template, 10.0 µl of Power SYBR green PCR master mix (Life Technologies), 1.0 µl of forward primer, and 1.0 µl of reverse primer. The 'two-step' cycling conditions were an initial heating at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min, and a final heating at 95°C for 15 s. The cycle threshold (C_t) value of each reaction was recorded as the expression level. The expression level of each gene for each isolate was calculated as the mean C_t value of the three technical replicates, when the standard error of the three C_t values was less than 0.5. The mean C_t values of *Cyp51* and *ScAtrG* were standardized by the mean C_t values of the internal control *Actin* to determine the relative expression level of *Cyp51* and *ScAtrG*. All steps from isolate growth to real-time PCR were repeated a second time. Analysis of variance was conducted using JMP Pro v11 (SAS Institute, Cary, NC) to identify significant differences in expression level between tebuconazole-resistant and tebuconazole-sensitive isolates.

Results

Differences in tebuconazole sensitivity of *Stagonosporopsis* species.

All 303 *S. citrulli* isolates collected from the southeastern United States in 2013 and 2014 were found to be sensitive to tebuconazole (Table 1). All *S. caricae* isolates from the southeastern United States were moderately ($n = 3$) or highly ($n = 16$) resistant to tebuconazole (Table 1). The *S. caricae* isolate from Brazil was highly resistant to tebuconazole. All other GSB or papaya fruit rot *Stagonosporopsis* isolates assayed were sensitive to tebuconazole, including nine *S. caricae* isolates originally collected from outside the southeastern United States.

***Cyp51* gene sequence and primer pairs.** The numbers of contigs with a minimum length of 200 bp varied from 2,275 in *S. caricae* isolate RG3 to 7,991 in *S. cucurbitacearum* isolate GSB26. The *Cyp51* gene was 1,676 bp in length (Fig. 1) and contained three exons (246, 198, and 1,134 bp) and two introns (50 and 48 bp) (GenBank accessions KU847917 to KU847921). The *Cyp51* genes identified in the GSB or papaya fruit rot fungi and *D. exigua* were homologous to *Cyp51B* of *G. zeae* and *A. fumigatus*. The homologs of *Cyp51A* of *G. zeae* and *A. fumigatus* were not detected in the nine draft genomes or the complete genome of *D. exigua*. A total of 1,314 bp of the upstream promoter region was sequenced.

***Cyp51* sequence variation among *S. caricae*.** The *Cyp51* genes from 11 highly tebuconazole-resistant *S. caricae* isolates, three moderately resistant isolates and two tebuconazole-sensitive isolates were sequenced and were compared with sequences from the draft genomes of three tebuconazole-sensitive *S. caricae* isolates and two additional tebuconazole-sensitive isolates. There were five different haplotypes among the *S. caricae* isolates (Fig. 1). The upstream promoter region, the first exon, and both introns were identical among the haplotypes. Tebuconazole-sensitive isolates were represented by haplotypes 1 and 2. Haplotype 1 (GenBank accession KU847917), which had two synonymous substitutions in the third exon, was shared by two tebuconazole-sensitive isolates (C166 and ATCC5257). Haplotype 2 (GenBank accession KU847918), shared by nine highly tebuconazole-resistant isolates (GA6057, GA6081, GA8007, GA8016, GA8036, GA8051, GA8061, GA8091, and GA8098) and three sensitive isolates (RG3, CBS102399, and CSB248.90), was the most common. Highly resistant isolate GA8002 had haplotype 3 (GenBank accession KU847919), which contained a nonsynonymous substitution in the second exon (isoleucine [I] to threonine [T] at codon position 113). Haplotype 4 (GenBank accession KU847920) was shared by highly resistant isolate 2245 and moderately resistant isolate GA8025 and contained a nonsynonymous substitution in the third exon (glycine [G] to serine [S] at codon position 463). The other two moderately resistant isolates (GA6011 and GA6075) shared haplotype 5 (KU827921), which had one nonsynonymous substitution (alanine [A] to glycine [G] at codon position 464) in the third exon. None of the substitutions was associated with tebuconazole resistance.

No difference in expression levels of *Cyp51* and *ScAtrG* between resistant and sensitive isolates. Using the expression level of *Actin* as an internal control, the induced expression levels of

Cyp51 and *ScAtrG* for isolates were calculated by averaging the two experimental replicates, since there was no significant difference between experiments ($P = 0.305$ for *Cyp51* and $P = 0.519$ for *ScAtrG*) or isolate by experiment interaction ($P = 0.539$ for *Cyp51* and $P = 0.263$ for *ScAtrG*). For *Cyp51*, the relative expression levels among tebuconazole-resistant *S. caricae* ($\bar{x} = 1.077$), tebuconazole-sensitive *S. caricae* ($\bar{x} = 1.003$), and tebuconazole-sensitive *S. citrulli* ($\bar{x} = 1.057$) and *S. cucurbitacearum* ($\bar{x} = 0.993$) were not significantly different (Fig. 2A). There were no significant differences in expression levels between the three resistant *S. caricae* isolates and the moderately resistant *S. caricae* isolate. In addition, the average expression levels of the ABC transporter *ScAtrG* for the four isolates were also similar, with no significant differences detected among tebuconazole-resistant *S. caricae* ($\bar{x} = 1.307$), tebuconazole-sensitive *S. caricae* ($\bar{x} = 1.354$), and tebuconazole-sensitive *S. citrulli* ($\bar{x} = 1.080$) and *S. cucurbitacearum* ($\bar{x} = 1.287$) isolates (Fig. 2B).

Discussion

Tebuconazole, a widely used triazole fungicide, is considered very effective in the management of GSB of cucurbits in the southeastern United States (Keinath and Hansen 2013; Thomas et al. 2012b). However, our identification of resistant isolates collected from watermelon fields in Georgia and observations of resistance in a field trial in Georgia (unpublished data) indicate that the efficacy of triazole fungicides in the southeastern United States could decline in the next few years. In a tebuconazole fungicide sensitivity assay, we identified 16 highly resistant (two from Tift County in 2013 and 14 from Cook County in 2014) and three moderately resistant (two from Tift County in 2013 and one from Cook County in 2014) GSB isolates. One isolate from Brazil was also identified as highly resistant. All of the tebuconazole-resistant GSB isolates identified from the southeastern United States and Brazil were *S. caricae* and all *S. caricae* isolates from the southeastern United States were highly resistant or moderately resistant to tebuconazole. However, isolates of *S. caricae* sampled from watermelon in California, cucumber in Michigan, a cucurbit in Brazil, and papaya in Hawaii, Brazil, and Chile were sensitive to tebuconazole (Table 1), which indicates that *S. caricae* is not simply a more-resistant species overall. Although the date when the sensitive *S. caricae* isolates were sampled is unknown, they were all obtained prior to 2013 when tebuconazole resistance was first detected in isolates from Georgia. *Stagonosporopsis citrulli*,

the dominant species causing GSB in the southeastern United States (Stewart et al. 2015), remains sensitive to tebuconazole. The three isolates of *S. cucurbitacearum* collected from regions outside the southeastern United States were also sensitive to tebuconazole.

Although *S. citrulli* and *S. caricae* are morphologically indistinguishable, distinct differences in tebuconazole sensitivity are evident

Fig. 2. Expression levels of **A**, *Cyp51* and **B**, *ScAtrG* relative to *Actin* among gummy stem blight fungi after growing on media amended with tebuconazole (0.3 $\mu\text{g/ml}$) for 7 days. Error bars indicate the standard error of the four biological replicates of tebuconazole-resistant and four tebuconazole-sensitive *Stagonosporopsis caricae* isolates each averaged over two experiments, as well as two biological replicates of tebuconazole-sensitive *S. citrulli* and *S. cucurbitacearum* isolates averaged over two experiments. Analysis of variance was conducted among four tebuconazole-resistant and four tebuconazole-sensitive isolates of *S. caricae*. The P value is shown to the left of the dashed reference line.

Fig. 1. Schematic of the *Cyp51* gene and upstream region of five genotypes from five tebuconazole-sensitive, 11 highly tebuconazole-resistant, and three moderately tebuconazole-resistant *Stagonosporopsis caricae* isolates. Total number of isolates (N), and number of sensitive (S), highly resistant (HR), and moderately resistant (MR) isolates with each genotype is shown. Black rectangles represent exons and arrows indicate the direction of transcription. Letters below the bars indicate polymorphic sites among the five genotypes. The amino acid locations of the putative protein for nonsynonymous substitutions are indicated above the bar.

between these two species causing GSB in the southeastern United States. The decline in efficacy of MBC (Keinath and Zitter 1998), QoI (Keinath 2009; Stevenson et al. 2004), and SDHI (Keinath 2012; Thomas et al. 2012a) fungicides against GSB fungi resulted from increased frequency of resistant isolates due to strong selection pressure from each fungicide. It is not clear how or from where the tebuconazole-resistant *S. caricae* populations arose in the southeastern United States (Li and Brewer 2016), but as tebuconazole use for GSB continues, it is possible that selection for resistant isolates of *S. caricae* with greater fitness could result in changes in the species composition of GSB fungi in the southeastern United States. Alternatively, tebuconazole-resistant *S. caricae* populations may be increasing as a result of introductions on cucurbit seed or from papaya.

We investigated the underlying genetic basis of tebuconazole resistance within *S. caricae* by comparing the nucleotide sequence of *Cyp51* in resistant and sensitive isolates. Triazole molecules bind to lanosterol 14 α -demethylase (Kelly and Kelly 2013), and, therefore, a single-site mutation in *Cyp51* causing an amino acid change in the enzyme is responsible for triazole resistance in several plant-pathogenic fungi (Cools et al. 2011; Frenkel et al. 2015; Schmitz et al. 2014). We sequenced and compared the coding region (1,676 bp) and promoter region (1,314 bp) of *Cyp51* from 11 highly resistant, three moderately resistant, and five sensitive *S. caricae* isolates. The promoter regions were compared because insertions were found in the promoter regions of *Cyp51* in other triazole-resistant plant pathogens (Cools et al. 2012; Schnabel and Jones 2001) that were responsible for increasing transcription. However, the promoter region, both introns, and the first exon were identical among sequences for all 19 isolates of *S. caricae* that were compared. Although there were polymorphic sites in the second and third exons, none of the haplotypes was clearly associated with the tebuconazole-resistant phenotype. Moreover, the most common haplotype was shared among resistant and sensitive isolates. Frequently reported mutational sites and amino acid substitutions identified in several other plant-pathogenic fungi, e.g., Y136F (Cools et al. 2013; Price et al. 2015), were not found in any of the tebuconazole-resistant *S. caricae* isolates. Thus, triazole resistance in *S. caricae* in the southeastern United States is not likely due to mutations in the *Cyp51* gene or upstream promoter region.

Increased expression of *Cyp51* is another mechanism for triazole resistance in fungi that we investigated in *S. caricae*. In addition to insertions in the promoter region of *Cyp51* leading to increased expression (Cools et al. 2012; Schnabel and Jones 2001), other unknown transcriptional enhancing factors in triazole-resistant isolates may act on the promoter region of *Cyp51* (Ma and Tredway 2013). After growing on tebuconazole-amended media for 7 days, the average expression levels of *Cyp51* in tebuconazole-resistant *S. caricae*, tebuconazole-sensitive *S. caricae*, and other tebuconazole-sensitive GSB fungi were not significantly different; therefore, gene duplication of *Cyp51* and other unknown transporters may be responsible for the tebuconazole resistance of *S. caricae*.

Differences in expression of some transporters, which are integral proteins that move molecules including nutrients and secrete substrates such as toxic compounds across membranes (Perlin et al. 2014), has been identified as a mechanism of triazole fungicide resistance. Transporters in the ABC and MFS superfamilies are responsible for decreasing fungicide accumulation within cells of many pathogenic fungi (Price et al. 2015). The ABC transporter homolog *ScAtrG*, identified and investigated in *S. caricae* in this study, did not show significant increases in expression in tebuconazole-resistant isolates. Homologs of other transporters that could be involved in fungicide efflux from the cell were not readily identified in the draft genome sequence of *S. caricae*, but it is possible that an unidentified transporter or transporters may contribute to triazole resistance.

Another mechanism for triazole resistance identified in fungi is multiple copies of *Cyp51* genes. Paralogs of *Cyp51*, designated as *Cyp51A* and *Cyp51B*, were first identified in the human pathogen *Aspergillus fumigatus* (Mellado et al. 2001). Both genes belonged to the lanosterol 14 α -demethylase family. In a study of plant-pathogenic fungi, Yin et al. (2009) found that tebuconazole induced the expression of both *Cyp51A* and *Cyp51B* in *Fusarium asiaticum* and

F. graminearum and determined that the sequences of *Cyp51A* were different between the two species. In another study of *F. graminearum* populations, polymorphism was found among *Cyp51A* sequences but not among *Cyp51B* sequences (Talas and McDonald 2015). *Cyp51* homologs identified in *D. exigua* and the genomes of the three *Stagonosporopsis* species in this study were orthologous to the more conserved *Cyp51B* of *A. fumigatus* and *F. graminearum*. Paralogs of *Cyp51B*, including *Cyp51A*, were not identified within the genomes of *D. exigua* (Mellado et al. 2001; Yin et al. 2009) or the three *Stagonosporopsis* species causing GSB. Interestingly, in recent studies of *Rhynchosporium commune*, three paralogs were identified, including *Cyp51A*, *Cyp51B*, and a pseudogene of *Cyp51A* designated as *Cyp51A-p*, and the presence of *Cyp51A* was associated with triazole resistance (Brunner et al. 2016). Paralogs of *Cyp51*, including *Cyp51A*, could be present in resistant isolates of *S. caricae* and serve as a possible mechanism for tebuconazole resistance. The three draft genomes of *S. caricae* searched for *Cyp51* homologs were all generated from triazole-sensitive isolates, so it would be worthwhile to sequence the genome of a resistant isolate to scan for paralogs.

Although the one or more molecular mechanisms for resistance to triazoles in *S. caricae* is not clear, we can rule out some of the known resistance mechanisms identified in other fungi, and we now know that the underlying basis of resistance is more complex than originally hypothesized. Studies investigating additional mechanisms are warranted because knowledge of the molecular basis of resistance will inform us of the evolutionary processes leading to triazole resistance in GSB fungi and, possibly, allow us to develop a molecular marker to quickly and accurately monitor triazole resistance in field populations. Future approaches could include RNA-seq to identify genes that are expressed at higher levels in resistant isolates or if paralogs of *Cyp51* are expressed. Recent work on *Cercospora beticola* identified 110 genes that were expressed at different levels in triazole-resistant isolates (Bolton et al. 2015). Until the molecular basis of resistance to triazoles is identified, the PCR-based marker for distinguishing *Stagonosporopsis* species causing GSB (Brewer et al. 2015) can be used to screen for *S. caricae*, which is more likely to be triazole-resistant and resistance can then be tested by fungicide sensitivity assays.

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