Molecular Mechanisms Involved in Qualitative and Quantitative Resistance to the Dicarboximide Fungicide Iprodione in *Sclerotinia homoeocarpa* Field Isolates

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Accepted for publication 14 September 2016.

ABSTRACT

The dicarboximide fungicide class is commonly used to control *Sclerotinia homoeocarpa*, the causal agent of dollar spot on turfgrass. Despite frequent occurrences of *S. homoeocarpa* field resistance to iprodione (dicarboximide active ingredient), the genetic mechanisms of iprodione resistance have not been elucidated. In this study, 15 field isolates (seven suspected dicarboximide resistant, three multidrug resistance (MDR)-like, and five dicarboximide sensitive) were used for sequence comparison of a histidine kinase gene, *Shos1*, of *S. homoeocarpa*. The suspected dicarboximide-resistant isolates displayed nonsynonymous polymorphisms in codon 366 (isoleucine to asparagine) in *Shos1*, while the MDR-like and sensitive isolates did not. Further elucidation of the *Shos1* function, using polyethylene glycol-mediated protoplast transformation indicated that *S. homoeocarpa*

mutants (*Shos1*^{I366N}) from a sensitive isolate gained resistance to dicarboximides but not phenylpyrrole and polyols. The deletion of *Shos1* resulted in higher resistance to dicarboximide and phenylpyrrole and higher sensitivity to polyols than *Shos1*^{I366N}. Levels of dicarboximide sensitivity in the sensitive isolate, *Shos1*^{I366N}, and *Shos1* deletion mutants were negatively correlated to values of iprodione-induced expression of *ShHog1*, the last kinase in the high-osmolarity glycerol pathway. Increased constitutive and induced expression of the ATP-binding cassette multidrug efflux transporter *ShPDR1* was observed in six of seven dicarboximide-resistant isolates. In conclusion, *S. homoeocarpa* field isolates gained dicarboximide resistance through the polymorphism in *Shos1* and the overexpression of *ShPDR1*.

Dollar spot, caused by *Sclerotinia homoeocarpa* (F. T. Bennett), is one of the most common and economically important diseases of cool-season turfgrass species worldwide (Smiley et al. 2005). Proper cultural management practices to turfgrass are often ineffective for complete dollar spot control. Therefore, multiple fungicide applications are often needed throughout the growing season to manage dollar spot and maintain high turf quality. However, repeated use of fungicides have led to the development of fungicide resistance in *S. homoeocarpa* populations (Smiley et al. 2005). Resistance to the benzimidazole and dicarboximide fungicide classes and reduced sensitivity to the sterol demethylation inhibitor (DMI) class have been reported in *S. homoeocarpa* field populations across North America (Cole et al. 1968; Detweiler et al. 1983; Jo et al. 2006; Miller et al. 2002; Popko et al. 2012; Putman et al. 2010). Resistance mechanisms for benzimidazole and DMI fungicides were previously elucidated in *S. homoeocarpa* (Hulvey et al. 2012; Koenraadt et al. 1992; Sang et al. 2015), whereas the one or more genetic mechanisms of dicarboximide resistance remain poorly understood.

Dicarboximide fungicides were initially introduced to control *Botrytis cinerea* in the late 1970s (Cui et al. 2002) and are now used to control a variety of *Sclerotinia* spp., *Monilinia* spp., and *Alternaria* spp. (Detweiler et al. 1983; Hutton 1988; Yoshimura et al. 2004). Iprodione and vinclozolin are two dicarboximide-active ingredients that have been registered to control dollar spot for the last three decades and played an important role in controlling

benzimidazole- and DMI-resistant populations (Detweiler et al. 1983). Dicarboximide field resistance has been reported in many plant-pathogenic fungi, such as *B. cinerea* (Beever and Brien 1983; Leroux et al. 2002; Katan 1982), *Monilinia fructicola* (Ritchie 1983), *Sclerotium cepivorum* (Littley and Rahe 1984), and *Alternaria alternata* (Hutton 1988). Genetic mechanisms responsible for dicarboximide resistance in *B. cinerea* have been well-documented and were associated with the amino acid changes in the putative target gene of iprodione, *Bos1*, which encodes a class III histidine kinase (HK) involved in osmotic and oxidative stress (Ma et al. 2007). In parallel, laboratory-induced iprodione-resistant mutants have shown cross-resistance to a phenylpyrrole fungicide, fludioxonil, and hypersensitivity to osmotic stress. Both iprodione and fludioxonil are known to affect the osmotic signal transduction cascade, but involvement of the mutations in *Bos1* in the cascade remains unresolved (Fillinger et al. 2012).

The first occurrence of iprodione field resistance in *S. homoeocarpa* was confirmed in the early 1980s by Detweiler et al. (1983). Since then, *S. homoeocarpa* isolates exhibiting resistance or reduced sensitivity to iprodione in monitored studies have been identified in the United States and Italy (Bishop et al. 2008; Jo et al. 2006; Mocioni et al. 2011; Putman et al. 2010). Putman et al. (2010) sampled 1,352 isolates from golf courses in the New England region and reported iprodione sensitivity to be quantitatively distributed for the majority of isolates but also reported a small number of “outlier” isolates with significantly reduced in vitro sensitivity levels (Putman et al. 2010). Despite reports of one or both qualitative or quantitative resistance to iprodione in *S. homoeocarpa*, the molecular mechanisms of resistance remain largely unknown. Furthermore, dicarboximide resistance mechanisms conferred by qualitative (disruptive or discrete) and quantitative (multistep or continuous) resistance within the same fungal pathogen have not been investigated.

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*The e-Xtra logo stands for “electronic extra” and indicates that one supplementary figure and one supplementary table are published online.

To examine genetic factors that contribute to a wide range of *S. homoeocarpa* iprodione sensitivity, we mined the osmosensing HK gene *Shos1* from transcriptomic data previously generated by Hulvey et al. (2012). The *Shos1* gene shares significant sequence similarity with the *Bos1* gene from *B. cinerea*, *Mfos1* gene from *Monilinia fructicola*, and *Shk1* gene from *Sclerotinia sclerotiorum*, which have all been known to play an important role in mediating qualitative resistance to iprodione (Duan et al. 2013; Ma et al. 2006, 2007). Additionally, an ATP-binding cassette (ABC) transporter, *ShPDR1*, in *S. homoeocarpa* was previously reported to be associated with mediating quantitative resistance to iprodione (Sang et al. 2015). Sang et al. (2015) reported that *S. homoeocarpa* field isolates with reduced in vitro sensitivity to propiconazole, boscalid (SDHI: succinate dehydrogenase inhibitor), and iprodione displayed constitutive overexpression of two ABC transporters (*ShatrD* and *ShPDR1*) and only *ShPDR1* expression was induced in response to iprodione. *ShPDR1* complemented a yeast ABC transporter deletion mutant in the presence of the aforementioned three chemicals, using a yeast heterologous expression system (Sang et al. 2015). However, the direct function of the *ShPDR1* gene in regard to dicarboximide resistance in *S. homoeocarpa* has not been elucidated by means of genetic transformation.

The main goal of this study was to investigate the genetic determinants explaining a wide range of iprodione sensitivity using *S. homoeocarpa* field isolates, which were sampled from the aforementioned published studies (Detweiler et al. 1983; Putman et al. 2010; Sang et al. 2015). The resistance determinants were validated using a genetic transformation system based on a split-marker approach, which was developed for the first time in *S. homoeocarpa*. The specific objectives were i) to sequence the *Shos1* gene of *S. homoeocarpa* field isolates for potential polymorphisms associated with qualitative resistance to iprodione, ii) to quantify in vitro sensitivities of isolates to iprodione, fludioxonil, propiconazole, and osmotic stress, iii) to analyze the relative expression (RE) of *ShPDR1* in isolates before and after iprodione exposure, and iv) to validate the function of the genes by generating *Shos1*^{1366N}, *Shos1*-knockout, and *ShPDR1* overexpression mutants from a sensitive *S. homoeocarpa* isolate and by quantifying *ShHog1* gene expression (last kinase of the high-osmolarity glycerol [HOG] pathway) with *Shos1*-generated mutants.

MATERIALS AND METHODS

Fungal isolates. The origins of the 15 *S. homoeocarpa* isolates used in this study are described below. One isolate (MI), which was previously confirmed to exhibit field resistance to iprodione (Detweiler et al. 1983), was collected from a creeping bentgrass putting green in Michigan. Six isolates with the highest iprodione EC₅₀ values (the mean effective concentration for reduction of 50% mycelial growth relative to the control) were selected from a total of 387 isolates assayed in a New England monitoring study (Putman et al. 2010). The origins of these six suspected dicarboximide-resistant isolates are as follows: CT34 (West Hartford, CT), CT45 and CT47 (Wethersfield, CT), CT91 (Enfield, CT), CT189 (Simsbury, CT), and NY2 (Newburgh, NY). In addition, eight isolates collected from four golf courses with prior fungicide exposure and one baseline location, all previously characterized in field efficacy, fungicide sensitivity, and fungicide resistance mechanism studies (Popko et al. 2012; Sang et al. 2015), were chosen. Isolates HRS10, SMS23, SMI27, JTS30, and HFS35 are considered dicarboximide-sensitive, based on previous in vitro fungicide sensitivity assays (Popko et al. 2012; Sang et al. 2015). Isolates WBI7, HRI11, and HFI40 are considered MDR-like isolates and have been previously confirmed to exhibit reduced sensitivities to three chemically unrelated fungicides, propiconazole, iprodione, and boscalid (Popko et al. 2012; Sang et al. 2015). All isolates were maintained on PDA (potato dextrose agar) (Difco

Laboratories, Detroit) slant tubes at 4°C until the study was performed.

DNA extraction, polymerase chain reaction (PCR) amplification and sequencing. Genomic DNA was extracted from all isolates, using the methods previously described by Saitoh et al. (2006) and Hulvey et al. (2012). The osmosensing HK gene *Shos1* (contig 4083; accession number JW817408) was mined from *S. homoeocarpa* transcriptomic data (BioProject, PRJNA167377) generated by Hulvey et al. (2012). Five primer sets were designed from *Shos1* to amplify and sequence the full-length of the *Shos1* gene (4,280 bp) for 10 isolates (CT34, CT45, CT91, CT189, NY2, MI, HRI11, HFS40, SMS23, and JTS30) and the six 1,490-bp HAMP repeats in the *Shos1* gene for five isolates (CT47, WBI7, HRS10, SMI27, and HFS40), using Primer3 (Rozen and Skaletsky 2000) (Supplementary Table S1). PCR reaction conditions followed the procedures of Sang et al. (2015). PCR amplicons were purified using ExoSAP-IT PCR cleanup reagent (Affymetrix, Santa Clara, CA) and were sequenced at the Genomic Resource Laboratory (University of Massachusetts, Amherst, MA).

Sequence and phylogenetic analyses. BLASTX and Pfam domain searches of the 3,942-bp *Shos1* coding sequence were conducted. Analyses of the full-length *Shos1* genomic sequence from 10 isolates and the six HAMP repeats of *Shos1* sequences from 15 isolates for an intron, and single nucleotide polymorphism (SNP) detection were performed using MEGA v5 (Tamura et al. 2011). The full-length amino acid sequences of *Shos1* and the reported osmosensing HK amino acid sequences of other species obtained from GenBank were used for the phylogenetic analysis. Amino acid sequences were aligned using MAFFT v7.110 with automated strategy selection and BLOSUM62 scoring matrix parameters (Kato and Toh 2008). The aligned amino acid sequences were imported into MEGA v5, and a neighbor-joining phylogenetic analysis was performed with the Poisson model, gamma distributed rates among sites, complete deletion of missing data, and 1,000 bootstrap replicates (Tamura et al. 2011).

In vitro fungicide sensitivity and osmotic stress assays. In vitro sensitivity assays for all 15 isolates were conducted on fludioxonil, iprodione, propiconazole, and NaCl-amended minimal medium (MM) (1 liter: 10 g of glucose, 1.5 g of K₂HPO₄, 2 g of KH₂PO₄, 1 g of (NH₄)₂SO₄, 0.5 g of MgSO₄·7H₂O, 2 g of yeast extract, and 12.5 g of agar), as per Sang et al. (2015). The iprodione and propiconazole EC₅₀ values of five dicarboximide-sensitive isolates and three MDR-like isolates previously used by Sang et al. (2015) were re-estimated in this present study. Isolates were previously stored on PDA slant tubes (4°C), were transferred to PDA, and were grown for 2 days at room temperature. Agar plugs (5 mm in diameter) were extracted from active colonies and were inoculated on the edge of nonamended MM plates; iprodione (0.01, 0.1, 1, 10, and 100 µg ml⁻¹), fludioxonil (0.001, 0.01, 0.1, 1, and 10 µg ml⁻¹), propiconazole (0.001, 0.01, 0.1, 1, and 10 µg ml⁻¹), and NaCl (0.5 and 1 M) amended MM plates. After 8 days of incubation, the diameter from agar plug to the colony margin was measured using 16EX digital calipers (Mahr, Göttingen, Germany). Two separate experiments and four replicates (petri plates) per each experiment were performed for each isolate or concentration. Iprodione, fludioxonil, and propiconazole EC₅₀ values and percent relative mycelial growth (RMG) of isolates on NaCl-amended medium were calculated as per Jo et al. (2006).

RNA extraction, cDNA synthesis, and relative quantification of *ShPDR1* and *ShHog1* expression. The ABC transporter *ShPDR1* was selected for this study, because overexpression of *ShPDR1* was positively correlated with reduced sensitivities to chemically unrelated fungicide classes, including dicarboximide (Sang et al. 2015). For analysis of *ShPDR1* expression levels, RNA samples were extracted from seven suspected dicarboximide-resistant isolates and two dicarboximide-sensitive isolates (SMS23 and JTS30) before and after exposure (1 h) to iprodione (1 µg ml⁻¹)

in this present study, using the methods detailed by Sang et al. (2015). Previously obtained C_T (cycle threshold) values of *ShPDR1* and actin gene (*Shact*) for three MDR-like (WBI7, HRI11, and HFI40) and three dicarboximide-sensitive isolates (HRS10, SMI27, HFS35) were used for the purpose of comparison (Sang et al. 2015). Expression of hog1-type mitogen-activated protein kinase *ShHog1*, mined from *S. homoeocarpa* genomic data (Bioproject, PRJNA302176), generated by Green et al. (2016), was also analyzed. RNA extraction was performed on the isolate and mutants in the absence and presence of iprodione ($10 \mu\text{g ml}^{-1}$) and propiconazole ($1 \mu\text{g ml}^{-1}$) for 1 h. cDNA synthesis from each sample of RNA was performed with the QuantiTect reverse transcription kit (Qiagen Inc., Valencia, CA). For relative quantification of *ShPDR1* and *ShHog1* expression, the actin gene (*Shact*) was selected as a housekeeping gene. The primers for *ShPDR1* and *Shact* were described by Sang et al. (2015). Relative gene expression was calculated using the comparative C_T method (Livak and Schmittgen 2001). All experiments were performed with two biological replicates and three technical replicates per each biological replicate for each isolate and treatment.

Construction of vectors for *Shos1*-knockout, *Shos1*^{1366N}, and overexpression of *ShPDR1*. The hygromycin resistance cassette PtpC-hph was amplified from pYHN3 (Rech et al. 2007), using F_ptpC and R_hph, and was cloned into pCR2.1-TOPO (Invitrogen, Carlsbad, CA), resulting in the plasmid Topo-hph. A 1-kb upstream flanking sequence of *Shos1* was amplified from genomic DNA (gDNA) of isolate HRS10, using primer F_*KpnI*_upos1 and R_*SpeI*_upos1. The amplicon was inserted into the *KpnI* and *SpeI* sites of the plasmid Topo-hph to generate the plasmid Topo-hph-upos1. Subsequently, a 1-kb downstream flanking sequence of *Shos1* amplified from gDNA of isolate HRS10 using the primers F_*NotI*_downos1 and R_*ApaI*_downos1

was inserted into *NotI* and *ApaI* sites of the plasmid Topo-hph-upos1 to generate the plasmid Topo- Δ Shos1. As a result, the *Shos1* deletion vector Topo- Δ Shos1 was constructed by inserting 1 kb of two flanking sequences of the *Shos1* gene into both sides of PtpC-hph in the Topo-hph vector. Two constructs were amplified with primers F_upos1/R_YG and F_HY/R_downos1, respectively, each containing 1 kb of upstream flank of the *Shos1* and 731 bp of hph or 1 kb of downstream flank of the *Shos1* and 1,126 bp of PtpC-hph (Fig. 1A). Two constructs were gel-purified using the Zymoclean gel DNA recovery kit (Zymo Research, Irvine, CA). For construction of *Shos1*^{1366N} vector, the full length of *Shos1* gDNA from dicarboximide-resistant isolates CT45 was amplified, using a primer set F_*KpnI*_Shos1 and R_*SpeI*_Shos1. The purified *Shos1* fragment was inserted into *KpnI* and *SpeI* sites of the plasmid Topo- Δ Shos1 to generate the plasmid Topo-*Shos1*^{1366N}. Both constructs were amplified with primers, F_Shos1/R_YG and F_HY/R_downos1, respectively.

To construct a gene overexpression plasmid for *S. homoeocarpa* (pYHN3-pShEF1 α), the plasmid pYHN3 (Rech et al. 2007) was modified by insertion of a multiple cloning site from pYES2 (Invitrogen, Carlsbad, CA), using primer set F_*NcoI*_MCS and R_*SpeI*_MCS and 1 kb of the upstream region of elongation factor 1 alpha (ShEF1 α) from gDNA of isolate HRS10, using primer set F_*ApaI*_ShpEF1 α and R_*NcoI*_ShpEF1 α . The full length of *ShPDR1* coding region amplified from plasmid pYES2-ShPDR1 (Sang et al. 2015) using F_*KpnI*_PDR1 and R_*SpeI*_PDR1 was inserted into the vector pYHN3-pShEF1 α , which resulted in a *ShPDR1* overexpression vector pYHN3-pShEF1 α -ShPDR1.

Protoplast preparation and transformation of *S. homoeocarpa*. The method for protoplast preparation and transformation of *S. homoeocarpa* was modified from Rollins (2013). In brief, mycelia of *S. homoeocarpa* isolate HRS10 were grown on PDA for 4 days

Fig. 1. Generation and validation of *Shos1*-knockout mutants, HRS10(Δ Shos1)-1, -2, and -3 from the *Sclerotinia homoeocarpa* isolate HRS10. **A**, Gene knockout strategy for *Shos1*. Short arrows indicate primer binding sites. **B**, Primer pair F2_Shos1/R3_Shos1 was used to amplify the partial region of *Shos1* gene (1,700 bp). **C**, Primer pair F_ptpC/R_hph was used to amplify the hygromycin resistance cassette (ptpC-hph) (1,391 bp). **D**, Primer pair F_detect_upos1/R_YG was used to amplify the left homologous arm with a partial fragment of the connecting area (2,021 bp). **E**, Primer pair F_HY/R_detect_downos1 was used to amplify the right homologous arm with a partial fragment of the connecting area (2,270 bp). M denotes molecular mass markers of the 1-kb ladder.

and were then inoculated in a 50-ml falcon tube (USA Scientific Inc., Ocala, FL) containing 25 ml of potato dextrose broth (Becton Dickinson, Franklin Lakes, NJ) and were incubated for 4 days at 23°C, without agitation. The mycelia were collected from four layers of sterile cheesecloth in a funnel, were washed with sterile water and then with protoplast buffer (0.8 M $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2 M $\text{C}_6\text{H}_5\text{Na}_3\text{O}_7 \cdot 2\text{H}_2\text{O}$, pH 5.5). The washed mycelia were cut into smaller pieces and were placed into a sterile 250-ml Erlenmeyer flask. Lysing enzymes (200 mg) from *Trichoderma harzianum* (Sigma-Aldrich, St. Louis) were dissolved in 3 ml of buffer (1 M sorbitol, 50 mM sodium citrate, pH 5.8), and 17 ml of protoplast buffer was added to the mycelium-containing Erlenmeyer flask. The flask was incubated at 28°C, with 100 rpm of agitation on a shaker for 3 h. The solution containing protoplasts was filtered through sterile cheesecloth and was collected in a sterile 250-ml Erlenmeyer flask. Next, 30 ml of 0.6 M KCl was added to the cheesecloth and was collected in the flask containing protoplast. The protoplast solution was transferred into a 50-ml falcon tube and was centrifuged for 10 min at 4°C ($3,000 \times g$). The pelleted protoplasts were washed twice with 10 ml of STC buffer (1 M sorbitol, 50 mM Tris, pH 8, 50 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$). Protoplast solution (500 μl) (1×10^7 protoplasts per milliliter in STC buffer), 6.25 μl of dimethyl sulfoxide, 31.25 μl of heparin (5 mg ml^{-1} prepared in STC buffer), and 125 μl of polyethylene glycol (PEG) solution (40% PEG 3350, 50 mM Tris, pH 8, 0.6 M KCl, 50 mM CaCl_2) were added to a 1.5-ml microcentrifuge tube and were then stored at -80°C .

For the transformation of *S. homoeocarpa*, protoplasts stored at -80°C were thawed on ice and 2 μg of split marker construct or 4 μg of plasmid was added to the tube. The tube was incubated on ice for 60 min, and then, 1 ml of PEG solution was added. The mixture was incubated at room temperature for 20 min. The protoplasts were transferred into a 15-ml screw-cap falcon tube (USA Scientific Inc.) and 5 ml of regeneration medium (239.6 g of sucrose, 0.5 g of yeast extract per liter) were added. The tube was incubated for 12 to 16 h at room temperature on a Bigger Bill digital oscillator (Thermo Scientific Thermolyne, Waltham, MA) at 150 rpm. The tube was centrifuged at $2,500 \times g$ for 10 min at 4°C, and the supernatant was discarded. The protoplasts were resuspended with 200 ml of STC buffer in the tube and were spread on regeneration agar medium containing 100 $\mu\text{g ml}^{-1}$ of hygromycin B (Invitrogen, Carlsbad, CA). Upon selection on hygromycin media for 4 to 8 days at 23°C, transformants were obtained and then hyphal tip-transferred to PDA containing 100 $\mu\text{g ml}^{-1}$ of hygromycin B at least three times.

In vitro sensitivity assays and pathogenicity tests of Shos1-knockout, Shos1^{I366N}, and ShPDR1 overexpressed mutants. In vitro sensitivity assays were conducted on the two Shos1-knockout mutants [HRS10(ΔShos1)-1 and HRS10(ΔShos1)-2], two Shos1^{I366N} mutants [HRS10(Shos1^{I366N})-1 and HRS10(Shos1^{I366N})-2], two PDR1 overexpression mutants [HRS10(ShPDR1^{OX})-1 and HRS10(ShPDR1^{OX})-2], and four isolates (HRS10, HRI11, CT45 and NY2) with iprodione, vinclozolin, and fludioxonil. Shos1-knockout, Shos1^{I366N}, and ShPDR1 overexpression mutants and HRS10 were also used for in vitro sensitivity assays with NaCl, KCl, glucose, D-sorbitol, and H_2O_2 . An agar plug (diameter, 4 mm) from each isolate grown on PDA for 3 days was inoculated on nonamended MM, iprodione (1.5, 3, 15, and 25 $\mu\text{g ml}^{-1}$), vinclozolin (1 and 5 $\mu\text{g ml}^{-1}$), fludioxonil (0.1 $\mu\text{g ml}^{-1}$), NaCl (0.4 M), KCl (0.2 M), glucose (1 M), D-sorbitol (1.2 M), and H_2O_2 (10 M) amended MM. For the calculation of RMG (%) value, the agar plug from each isolate was also inoculated on the individual MM. After the plates had been incubated at 25°C, RMG of each isolate was measured after 42, 62, and 144 h, and pictures were taken. The experiment was repeated three times.

For testing pathogenicity, creeping bentgrass (cv. Penncross) was grown in a greenhouse at 18°C day and 16°C night temperatures with a 12-h photoperiod and at 70% relative humidity. Six leaf blades from 2-month-old bentgrass plants were excised and were

placed on sterile water-saturated filter paper (Whatman, Maidstone, U.K.) in 9-cm petri dishes. An agar plug (4 mm in diameter) extracted from active mycelial colonies of *S. homoeocarpa* isolates (HRS10, HRI11, and CT45) and Shos1-knockout, Shos1^{I366N}, and ShPDR1^{OX} transformants grown on PDA was placed on the center of leaf blades. Inoculated leaf blades were incubated at 23°C at 100% relative humidity in the culture room. The lesion diameter on individual leaf blades was measured 2 days after inoculation. The whole experiment was repeated twice.

Statistical analysis of in vitro sensitivity assay and quantitative PCR data. For statistical analysis of in vitro sensitivity, field isolates examined were categorized into three groups according to the presence or absence of I366 N mutation and overexpression of *ShPDR1* (Table 1). First, group 1 (dicarboximide-resistant isolates CT34, CT45, CT47, CT91, CT189, NY2, and MI), containing the I366 N mutation in *Shos1*, and groups 2 (MDR-like isolates WBI7, HRI11, and HFI40) and 3 (dicarboximide-sensitive isolates: HRS10, SMI27, SMS23, JTS30 and HFS35), not containing I366 N in *Shos1*, were compared. Furthermore, group 2, showing constitutive and induced overexpression of *ShPDR1*, and group 3, showing no constitutive and induced overexpression of *ShPDR1*, were compared. Analysis of variance (ANOVA) and Fisher's protected least significant difference (LSD) were conducted to compare mean EC_{50} and percent RMG values between the aforementioned groups. ANOVA was conducted on RE values for *ShPDR1* between group 1 and group 2 and between group 1 and group 3. Linear regression analysis was conducted with iprodione EC_{50} values and $\log_{10}$ -transformed mean RE values of *ShPDR1* constitutive expression for all 15 isolates.

ANOVA and Fisher's protected LSD were conducted to compare mean percent RMG values among 10 isolates (HRS10, HRI11, CT45, NY2, and two Shos1^{I366N}, two Shos1-knockout, and two ShPDR1 overexpression mutants), percent RMG values among four isolates (HRS10, HRI11, and two ShPDR1 overexpression mutants), percent RMG values among four isolates (HRS10, and Shos1^{I366N}, Shos1-knockout, ShPDR1 overexpression mutants), and mean lesion diameter among nine isolates (HRS10, HRI11, CT45, and two Shos1^{I366N}, two Shos1-knockout, and two ShPDR1 overexpression mutants). Last, ANOVA was conducted on fold induction values for *ShHog1* expression between HRS10 and the Shos1^{I366N} mutant and between HRS10 and the Shos1-knockout mutant. All statistical analyses were conducted using the JMP software package, version 10.0 (SAS Institute Inc., Cary, NC).

RESULTS

Sequence and phylogenetic analyses. The HK gene *Shos1* from *S. homoeocarpa* was characterized by BLAST search, Pfam domain, phylogenetic analyses, and sequencing to detect potential polymorphisms associated with dicarboximide resistance. A BLASTX search revealed that Shos1 shares high homology with three genes encoding a HK two-component regulatory system, *Shk1* from *Sclerotinia sclerotiorum* (accession number EDN97840), *Mfos1* from *Monilinia fructicola* (accession number ABF60145), and *Bos1* from *B. cinerea* (accession number AFQ90105). These genes have been reported to mediate resistance to the dicarboximide fungicide class (Cui et al. 2004, 2002; Duan et al. 2013; Ma et al. 2006, 2007; Oshima et al. 2002) and were identified by an E-value of 0 and coding sequence coverage of 99%. The Shos1 sequence shares 94% of amino acid identity with Shk1 (1,234 out of 1,310), Mfos1 (1,237 out of 1,317), and Bos1 (1,234 out of 1,316). Six HAMP repeat domains, one kinase domain, and one response regulator domain were detected by a Pfam domain search. Phylogenetic analysis showed that *Shos1*, *Shk1*, *Mfos1*, and *Bos1* are placed in the same clade and were supported by a high bootstrap value (100%) (Supplementary Fig. S1). The *Shos1* coding sequence from HRI11 was deposited in GenBank with accession number KM576215.

Comparison of sequences obtained from gDNA sequencing and transcriptomic data indicated that the 4,280 bp of *Shos1* is interrupted by six introns. The SNP detection analysis of the full length of *Shos1* gene from 10 isolates indicated that only polymorphisms at codon 366, located in the second repeat of HAMP domain of *Shos1*, are perfectly correlated to suspected dicarboximide-resistant isolates. Further sequence analysis of six

HAMP repeats (1,490 bp) in the *Shos1* gene from 15 isolates revealed that ATC at codon 366 in MDR-like and sensitive isolates was replaced by AAT in resistant isolates CT34, CT45, CT47, CT91, and CT189, and by AAC in resistant isolates MI and NY2. However, these altered nucleotides encoding the same amino acid change an isoleucine to an asparagine at codon 366 (I366 N) (Fig. 2).

TABLE 1. In vitro sensitivity of *Sclerotinia homoeocarpa* isolates to fungicides and NaCl and mean comparison of the sensitivity among three groups of isolates

Groups ⁴ /isolates	Amino acid substitution I366 N in Shos1	Overexpression of <i>ShPDR1</i> ^w	EC ₅₀ values (μg/ml) ^u			RMG (%) ^v	
			Iprodione ^x	Fludioxonil	Propiconazole ^x	0.5 M of NaCl	1 M of NaCl
Group 1 (dicarboximide-resistant)							
CT34	+	+	2.186	0.006	0.412	26.9	8.1
CT45	+	+	1.660	0.003	0.383	19.5	6.9
CT47	+	+	2.177	0.005	0.354	21.0	3.7
CT91	+	+	1.501	0.002	0.825	17.1	3.1
CT189	+	+	2.291	0.004	0.361	28.1	8.0
MI	+	+	2.097	0.008	0.318	12.7	2.1
NY2	+	–	1.310	0.003	0.140	21.2	1.4
Group 2 (multidrug resistance-like)							
HRI11	–	+	0.761	0.005	0.753	8.6	3.0
HFI40	–	+	0.757	0.002	0.604	17.8	4.6
WBI7	–	+	0.824	0.003	0.544	10.6	2.9
Group 3 (dicarboximide-sensitive)							
SMI27	–	–	0.550	0.002	0.199	6.7	2.0
HRS10	–	–	0.526	0.001	0.016	11.8	2.1
HFS35	–	–	0.540	0.001	0.025	14.1	5.0
JTS30	–	–	0.524	0.002	0.020	13.0	3.4
SMS23	–	–	0.398	0.001	0.068	9.0	1.4
Group 1 versus ^y			1.89 a	0.004 a	0.40	20.9 a	4.7
Groups 2 + 3			0.61 b	0.002 b	0.28	11.5 b	3.1
<i>P</i> value ^z			***	**	NS	***	NS
Group 2 versus			0.78 a	0.003 a	0.63 a	12.3	3.5
Group 3			0.51 b	0.001 b	0.07 b	10.9	2.8
<i>P</i> value ^z			***	**	***	NS	NS

ⁱ Field isolates were grouped based on presence (+) or absence (–) of amino acid substitution I366 N and overexpression of *ShPDR1* as follows: Group 1 (dicarboximide-resistant), Group 2 (multidrug resistance-like), and Group 3 (dicarboximide-sensitive).

^u The EC₅₀ value is the effective fungicide concentration inhibiting mycelial growth of the isolates on fungicide-amended media by 50% compared with growth on nonamended media.

^v Relative mycelial growth (RMG) (%) was calculated by dividing the mean colony diameter of isolate on minimal medium (MM) amended with 0.5 or 1 M of NaCl by the mean colony diameter of isolate grown on nonamended MM.

^w Isolates (+) showed a higher constitutive expression of *ShPDR1* than the control isolate (SMS23) and higher induced expression of *ShPDR1*.

^x Iprodione and propiconazole EC₅₀ values of Group 2 and Group 3 isolates adapted from Sang et al. (2015) were presented.

^y Means followed by the same letter are not significantly different according to Fisher's protected least significant difference test ($\alpha = 0.05$).

^z Asterisks (** and ***) represent significant difference at $P < 0.01$ and 0.001 , respectively. NS, not significant.

Fig. 2. A, Schematic diagram of the partial *Shos1* gene structure showing the location of primers used in this study. The vertical arrow indicates a substitution at amino acid position 366 of the *Shos1* gene in dicarboximide-resistant isolates of *Sclerotinia homoeocarpa*. **B**, Alignment of partial deduced amino acid sequence of *Shos1* gene from dicarboximide-resistant (Shos1-DR), multidrug resistance-like (Shos1-MDR), and dicarboximide-sensitive (Shos1-DS) isolates of *S. homoeocarpa*. The shaded letters indicate the change at amino acid position 366 correlated with resistance to iprodione in dicarboximide-resistant isolates. Asterisks indicate identical amino acids among the isolates compared.

Sensitivity of *S. homoeocarpa* isolates to fungicides and osmotic stress. The in vitro sensitivity of 15 individual field isolates to iprodione (dicarboximide), propiconazole (DMI), fludioxonil (phenylpyrrole), and NaCl (osmotic stress) were assayed and are presented in Table 1. Furthermore, mean EC₅₀ values among group 1 (dicarboximide-resistant), group 2 (multidrug resistance-like), and group 3 (dicarboximide-sensitive) were compared with correlate genetic determinants with their sensitivity levels. The mean iprodione EC₅₀ values for group 1 (isolates containing I366 N mutation in *Shos1*) were significantly higher than mean iprodione EC₅₀ values of groups 2 and 3 (isolates that did not contain I366 N mutation in *Shos1*). In addition, group 2 (isolates that overexpressed *ShPDR1*) had significantly higher mean iprodione EC₅₀ values than group 3 isolates. Although group 1 had statistically higher mean fludioxonil EC₅₀ values than combined groups 2 and 3, the EC₅₀ values were numerically low in all individual isolates and, most likely, were biologically insignificant (Table 1). The mean propiconazole EC₅₀ values in group 2 were significantly higher than the ones in group 3.

The mean percent RMG values in group 1 on 0.5 M NaCl amended MM were significantly higher than those of both groups 2 and 3; however, isolate MI in group 1 had a similar RMG value to group 3 isolates. There is no statistical difference among the three groups on 1 M NaCl amended MM (Table 1).

Quantitative reverse transcription (qRT)-PCR analysis of *ShPDR1*. To test the possible involvement of *ShPDR1* overexpression in iprodione resistance, mean RE values of *ShPDR1* in group 1 isolates were compared with mean RE values of groups 2 and 3. Group 1 (mean = 10.69 ± 0.68 standard error [SE]) showed significantly higher constitutive RE values of *ShPDR1* than group 3 (mean = 1.40 ± 0.72 SE) ($P < 0.0001$) but was not significantly different from group 2 (mean = 9.02 ± 1.15 SE) ($P = 0.25$). In the presence of iprodione (1 µg ml⁻¹), group 1 isolates exhibited increased RE of *ShPDR1*, with the exception of isolate NY2. In contrast, increased RE of *ShPDR1* was not observed in isolates JTS30 and SMS23 (group 3), behaving as the other dicarboximide-sensitive isolates previously characterized for *ShPDR1* expression (Sang et al. 2015) (Fig. 3).

Linear regression of *ShPDR1* expression and EC₅₀ values for iprodione. The linear regression analysis was performed to examine whether constitutive expression of *ShPDR1* is correlated

Fig. 3. Relative expression (RE) of *ShPDR1* in 15 field isolates before and after exposure to iprodione (1 µg ml⁻¹) for 1 h. Light and dark gray bars indicate mean constitutive RE values and mean iprodione-induced RE values, respectively. Error bars represent one standard error from the mean. For comparison purposes, *ShPDR1* expression values of multidrug resistance (MDR)-like and three dicarboximide-sensitive isolates adapted from Sang et al. (2015) were reanalyzed and presented.

with iprodione sensitivity. The linear relationship between log₁₀-transformed mean RE values for *ShPDR1* and EC₅₀ values of iprodione was significant ($P = 0.0058$). The dicarboximide-resistant isolates with both *ShPDR1* overexpression and *Shos1* mutation, except for isolate NY2, showed higher iprodione EC₅₀ values than MDR-like isolates with *ShPDR1* overexpression alone (Fig. 4).

Generation of *Shos1*-knockout, *Shos1*^{I366N}, and *ShPDR1* overexpression mutants of *S. homoeocarpa*. For functional validation of *Shos1* and *ShPDR1* genes, we generated *Shos1*-knockout, *Shos1*^{I366N}, and *ShPDR1* overexpression mutants from sensitive *S. homoeocarpa* isolate HRS10. Among 24 hygromycin-resistant transformants, three *Shos1*-knockout mutants were confirmed by PCR with four primer pairs. A 1,700-bp partial region in the *Shos1* gene was only amplified in isolate HRS10, using the primer pair F2_Shos1 and R3_Shos1 (Fig. 1B). A pair of primers, F_ptrpC and R_hph, were used to amplify a 1,391-bp region of PtrpC-hph, which was only detected in three *Shos1*-knockout mutants HRS10(Δ*Shos1*)-1, HRS10(Δ*Shos1*)-2, and HRS10(Δ*Shos1*)-3 (Fig. 1C). A 2,021-bp section of the left flanking region and a 2,270-bp section of right flanking region were amplified in three *Shos1*-knockout mutants, using two pairs of primers, F_detect_upos1/R_YG and F_HY/R_detect_downos1, respectively (Fig. 1D and E).

From 18 hygromycin-resistant transformants, three *Shos1*^{I366N} mutants were confirmed by the aforementioned method, using primer sets F_ptrpC/R_hph, F5_Shos1/R_YG, and F_HY/R_detect_downos1. The analysis of the full-length *Shos1* gene sequence from three *Shos1*^{I366N} mutants indicated that ATC at codon 366 in isolate HRS10 was replaced by AAT in the three mutants.

Two *ShPDR1* overexpression mutants that exhibited the highest RMG values on iprodione (1.5 µg ml⁻¹) were selected from 45 hygromycin-resistant transformants. The total RNA of two *ShPDR1* overexpression mutants, HRS10(*ShPDR1*^{OX})-1 and HRS10(*ShPDR1*^{OX})-2, and HRS10 were extracted for comparison of the RE of *ShPDR1*. The HRS10(*ShPDR1*^{OX})-1 and HRS10(*ShPDR1*^{OX})-2 mutants showed 3- to 5.6-fold increased expression of *ShPDR1* over the sensitive isolate HRS10.

In vitro sensitivity to fungicides, osmotic and oxidative stress, and pathogenicity tests of *Shos1*-knockout, *Shos1*^{I366N} and *ShPDR1* overexpression mutants of *S. homoeocarpa*. The previously described mutants and field isolates were assayed on different fungicide classes, osmotic, and oxidative stresses. The

Fig. 4. Relationship between log₁₀ relative expression (RE) values of *ShPDR1* and iprodione EC₅₀ values for 15 field *Sclerotinia homoeocarpa* isolates. Triangles denote dicarboximide resistant isolates (Group 1), diamonds denote multidrug resistance-like isolates (Group 2), and dots denote dicarboximide-sensitive isolates (Group 3).

HRS10(Δ Shos1)-1 and HRS10(Δ Shos1)-2 mutants, HRS10(Shos1^{I366N})-1 and HRS10(Shos1^{I366N})-2 mutants, and dicarboximide-resistant isolates (NY2 and CT45) showed resistance to iprodione at 3 μ g ml⁻¹ and vinclozolin at 1 μ g ml⁻¹ (Fig. 5A). Furthermore, Shos1-knockout mutants and CT45 containing the mutation in *Shos1* and overexpression of *ShPDR1* showed resistance to iprodione at 15 μ g ml⁻¹; however, only Shos1-knockout mutants showed resistance to iprodione at 25 μ g ml⁻¹, vinclozolin at 5 μ g ml⁻¹, and fludioxonil at 0.1 μ g ml⁻¹. Since Shos1-knockout and Shos1^{I366N} mutants and dicarboximide-resistant isolates grew quickly on media amended with 1.5 μ g ml⁻¹ of iprodione, only four strains per plate, i.e., HRS10(ShPDR1^{OX})-1 and HRS10(ShPDR1^{OX})-2, HRS10, and HRI11, were compared at this concentration. ShPDR1 overexpression mutants HRS10(ShPDR1^{OX})-1 and HRS10(ShPDR1^{OX})-2 and MDR-like isolate HRI11 showed reduced sensitivity to iprodione at 1.5 μ g ml⁻¹ (Fig. 5A). For osmotic and

oxidative stresses, only the Shos1-knockout mutants showed increased sensitivity to glucose at 1 M and sorbitol at 1.2 M (Fig. 5B).

In addition, the pathogenicity of mutants and field isolates mentioned above was assayed on detached leaf blades of creeping bentgrass. All isolates and mutants were able to cause infection on leaf blades, with average lesion size ranging from 19 to 22 mm 2 days after inoculation, but no statistical difference was observed ($P = 0.65$).

RE of *ShHog1* in Shos1-knockout and Shos1^{I366N} mutants. The function of Shos1 was further characterized by quantifying expression of *ShHog1* in Shos1 mutants and the sensitive isolate. The qRT-PCR assay indicated that *ShHog1* expression was up-regulated by iprodione treatment in HRS10 (sixfold increase), HRS10(Shos1^{I366N})-1 (4.4 fold increase), and HRS10(Δ Shos1)-1 (1.4 fold increase), which were statistically significant ($P < 0.0001$). Propiconazole treatment did not induce expression of *ShHog1* in

Fig. 5. Sensitivity of *Sclerotinia homoeocarpa* isolates to fungicides, osmotic stress, and oxidative stress. **A**, Sensitivity to different fungicides. 1, HRS10; 2, HRI11; 3, HRS10(ShPDR1^{OX})-1; 4, HRS10(ShPDR1^{OX})-2; 5, HRS10(Shos1^{I366N})-1; 6, HRS10(Shos1^{I366N})-2; 7, HRS10(Δ Shos1)-1; 8, HRS10(Δ Shos1)-2; 9, CT45; and 10, NY2. The pictures were taken after the isolates grew for 42 h on minimal medium (MM) without and with iprodione, vinclozolin, and fludioxonil. *The picture of plate with 1.5 μ g ml⁻¹ of iprodione were taken after 144 h. 1, HRS10; 2, HRI11; 3, HRS10(ShPDR1^{OX})-1; and 4, HRS10(ShPDR1^{OX})-2. **B**, Sensitivity to osmotic stress (generated by glucose, sorbitol, NaCl, and KCl) and oxidative stress (generated by H₂O₂). 1, HRS10; 2, HRS10(ShPDR1^{OX})-1; 3, HRS10(Shos1^{I366N})-1; and 4, HRS10(Δ Shos1)-1. The pictures were taken after the isolates were grown for 62 h on MM without and with glucose, sorbitol, NaCl, KCl, and H₂O₂.

HRS10 and HRS10(Δ Shos1)-1; however, *ShHog1* expression was up-regulated in HRS10(Shos1^{I366N})-1 by 1.6 fold (Fig. 6).

DISCUSSION

This study reports both qualitative and quantitative types of iprodione resistance in *S. homoeocarpa* field isolates, i.e., an amino acid substitution in the HK gene *Shos1* and the overexpression of the ABC transporter gene *ShPDR1*, respectively. Specifically, the isolates examined in this study with the highest iprodione EC₅₀ values contain the two aforementioned factors, and the MDR-like isolates exhibiting lower levels of iprodione resistance exhibit quantitative overexpression of the *ShPDR1* only. Our findings were validated by an analysis of Shos1^{I366N}, Δ Shos1, and *ShPDR1* overexpression mutants from a dicarboximide-sensitive isolate. The Shos1^{I366N} mutants exhibiting resistance to iprodione indicated that the mutation (I366N) is a qualitative determinant of iprodione resistance, and *ShPDR1* overexpression mutants revealed that the level of *ShPDR1* expression contributes to a quantitative deterrent of reduced iprodione sensitivity.

In dicarboximide field-resistant isolates, an amino acid substitution at codon 366 in *Shos1*, from a hydrophobic isoleucine to a hydrophilic asparagine, resulted in decreased hydrophobicity in the region of this residue. So, it is plausible that the binding affinity of dicarboximide fungicides to *Shos1* is affected directly. The I366 N mutation does not affect the sensitivity of *S. homoeocarpa* to fludioxonil and osmotic stress, which suggests the effect of mutation is likely specific to dicarboximides. On the other hand, the *Shos1* deletion mutants exhibited a higher level of resistance to dicarboximide fungicides than Shos1^{I366N} mutants and resistant to a fludioxonil fungicide as well. The resistant phenotype to both fungicides has been reported in class III HK deletion mutants in many other fungal species, *Neurospora crassa*, *Magnaporthe grisea*, *Cochliobolus heterostrophus*, *B. cinerea*, and *Sclerotinia sclerotiorum* (Duan et al. 2013; Fujimura et al. 2000; Motoyama et al. 2005; Schumacher et al. 1997; Viaud et al. 2006; Yoshimi et al. 2004). Additionally, the *Shos1*-knockout mutants showed a strong growth reduction on polyols (sorbitol and glucose) but not on salt (NaCl and KCl) (Fig. 5). The HK deletion mutants exhibited differing osmotic stress sensitivities based on fungal species (Duan et al. 2013); however, there is little understanding of the biological or ecological reasons regarding these differential osmotic stress sensitivities. For example, the mutants of *Magnaporthe oryzae* and *Fusarium oxysporum* show a higher sensitivity to polyol stress than to salt (Motoyama et al. 2005; Rispaill and Pietro 2010), but the mutants of *Neurospora crassa*, *Cochliobolus heterostrophus*, *B. cinerea*, and *Sclerotinia sclerotiorum* display increased sensitivity to salt stress rather than to polyols (Alex et al. 1996; Duan et al. 2013; Viaud et al. 2006; Yoshimi et al. 2004). For oxidative stress, gene inactivation of *Shk1* from *Sclerotinia sclerotiorum* confers hypersensitivity to H₂O₂ (Duan et al. 2013), whereas the HK deletion mutants in *B. cinerea* and *S. homoeocarpa* did not exhibit increased sensitivity to oxidative stress (Viaud et al. 2006) (Fig. 5). In addition, the HKs in *Fusarium oxysporum*, *B. cinerea*, and *Alternaria brassicicola* are required for full pathogenicity (Cho et al. 2009; Rispaill and Pietro 2010; Viaud et al. 2006) but not in *S. homoeocarpa*, like *Magnaporthe grisea* and *Sclerotinia sclerotiorum* (Duan et al. 2013; Motoyama et al. 2005). Although *S. homoeocarpa* is closely related to *Sclerotinia sclerotiorum* and *B. cinerea*, the function of HK in osmotic and oxidative stresses and virulence shares or differs among these fungal species.

The present study provided further insight on the mechanisms of differential sensitivity of Shos1^{I366N} and *Shos1*-knockout mutants to iprodione by quantifying the expression of *ShHog1*. The increased iprodione resistance level in the *Shos1* deletion mutant over the Shos1^{I366N} mutant was correlated with the decreased iprodione-induced *ShHog1* expression (Figs. 5 and 6). Since HK is the upstream factor in an osmotic signaling system, the mutants lacking HK do not

exhibit phosphorylation of HOG mitogen-activated protein kinase (MAPK) caused by fungicides (Tanaka and Izumitsu 2010; Yoshimi et al. 2005). In addition, translocation of phosphorylated Hog1 to the nucleus is preceded by the increased expression of the *Hog1* gene (Marques et al. 2006). The dicarboximide mode of action suggested by Tanaka and Izumitsu (2010) was that the fungicide targeting HK induces abnormal phosphorylation of the HOG1 MAPK, which alters downstream gene expression. Therefore, the *Shos1* deletion mutant may not affect expression or phosphorylation of *ShHog1* after exposure to iprodione, resulting in high resistance to iprodione. On the other hand, the Shos1^{I366N} mutant was still able to regulate *ShHog1* expression but less than the wild strain. Therefore, lower levels of improper *hog1*-downstream gene expression may occur in response to iprodione. In order to fully understand the function of the mutation (I366N) and HK in *S. homoeocarpa*, further studies on the manipulation of genes from the HOG pathway, such as homologous genes of *B. cinerea* (*BcSak1*, *BcOs4*, *BcOs5*, *BcRrg1*, and *BcSkn7*) (Liu et al. 2008; Segmüller et al. 2007; Yan et al. 2010, 2011; Yang et al. 2012, 2015) and downstream genes of the pathway are required.

From the data presented, six of seven dicarboximide-resistant isolates of *S. homoeocarpa* with the I366 N substitution in *Shos1* exhibited constitutive and induced overexpression of *ShPDR1*. Dicarboximide-resistant isolate NY2 had the lowest level of insensitivity to iprodione among dicarboximide-resistant isolates and did not exhibit induced expression of *ShPDR1* (Fig. 3). The Shos1^{I366N} mutants that did not overexpress *ShPDR1* also exhibited a lower iprodione resistance level than the iprodione-resistant isolate (CT45) that contained both resistance factors (Fig. 5). Furthermore, overexpression of *ShPDR1* in a sensitive isolate resulted in reduced sensitivity to iprodione (Fig. 5). These results suggest *ShPDR1* overexpression may play a role in the additive or quantitative resistance development in dicarboximide-resistant isolates but contributes less than the I366 N substitution in *Shos1*. However, additional studies are needed to test these hypotheses, using a larger and more diverse pool of dicarboximide-resistant field isolates.

This study and our previous studies, which have investigated the genetic determinants of MDR and DMI resistance (Hulvey et al.

Fig. 6. Relative expression (RE) of *ShHog1* in HRS10, HRS10(Shos1^{I366N})-1, and HRS10(Δ Shos1)-1 before and after treatment with iprodione (10 μ g ml⁻¹) and propiconazole (1 μ g ml⁻¹) for 1 h. White bars indicate mean constitutive RE values, light gray bars indicate mean iprodione-induced RE values, and dark gray bars indicate mean propiconazole-induced RE values. Error bars represent one standard error from the mean.

2012; Sang et al. 2015), indicate that *S. homoeocarpa* has evolved multiple mechanisms of resistance to a specific fungicide and multiple fungicides. Understanding the mechanisms of fungicide resistance in this pathogen contributes to the information for evolution of other sterile fungi. This study also illustrated the use of a PEG-mediated protoplast transformation system in *S. homoeocarpa* for the first time and this methodology could be utilized to study the genetics and biology of *S. homoeocarpa* with the recent release of transcriptomic and genomic sequences of *S. homoeocarpa* (Green et al. 2016; Hulvey et al. 2012; Orshinsky et al. 2012). Our findings indicate that either the I366 N mutation in *Shos1*, overexpression of *ShPDR1*, or both are conferring in vitro iprodione resistance but not pathogenicity; however, we have not confirmed to what extent these genetic determinants are associated with decreased iprodione field efficacy, which is under investigation. Findings from this study will be utilized for developing a molecular diagnostic tool for detection of iprodione resistance, which will help management of dollar spot on golf courses.

ACKNOWLEDGMENTS

We appreciate the generosity of A. I. Putman from University of California, Riverside for providing some of the *S. homoeocarpa* isolates used in this study. We thank our lab members, E. Allan, J. Stoner, and A. Ooi for careful review of this manuscript and thank V. Palanukorn for help with the protoplast preparation. This material is based upon work supported by the National Institute of Food and Agriculture (NIFA), United States Department of Agriculture (USDA), the Massachusetts Agricultural Experiment Station, and the Stockbridge School of Agriculture at the University of Massachusetts Amherst, under project number MAS00436. The contents are solely the responsibility of the authors and do not necessarily represent the official views of the USDA or NIFA.

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