Multidrug Resistance Conferred by Xenobiotic Detoxification in the Ascomycete Fungus Sclerotinia homoeocarpa

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ABSTRACT

Dollar spot is caused by Sclerotinia homoeocarpa and is the most economically significant disease on high amenity turfgrass. Repeated fungicide applications have been utilized to provide high quality turf. However, fungicide resistance has developed to the demethylation inhibitor (DMI), dicarboximides and benzimidazoles. S. homoeocarpa isolates with cross-resistance to different DMIs and multiple-resistance to different fungicides have been documented. Recently, multidrug resistance (MDR) is becoming more problematic in pathogenic fungi. Our studies indicate that a DMI insensitive field population of S. homoeocarpa exhibiting the MDR phenotype constitutively overexpressed two ATP-binding cassette (ABC) efflux transporters, ShatrD and ShPDR1. These transporters complemented a hypersensitive yeast mutant in the presence of multiple fungicides. We summarized our recent understanding of xenobiotic detoxification operated MDR, which was conferred by ABC transporters with substrate specificity. We also discussed the involvement of cytochrome P450 (CYP450) monooxygenase in xenobiotic detoxification, and furthermore the regulatory system of the ABC transporters and CYP450 regulated by a fungal specific-transcription factor in S. homoeocarpa. This is the first report of establishing a molecular mechanism for the regulation of antifungal/xenobiotic detoxification conferring multidrug resistance in plant pathogenic fungi.

INTRODUCTION

Dollar spot, caused by Sclerotinia homoeocarpa, is the most economically significant disease of turfgrasses (Vargas et al. 1992). Repeated fungicide applications are utilized throughout the growing season to provide high quality turf. However, fungicide resistance has developed to the demethylation inhibitor (DMI), dicarboximide and benzimidazole fungicide classes due to
repeated fungicide exposure (Detweiler et al. 1983; Jo et al. 2006; Popko et al. 2012). An improved understanding of the mechanisms of fungicide resistance is needed to formulate improved control strategies. Hulvey et al. (2012) and Sang et al. (2015) used transcriptomic and molecular tools to find genetic factors conferring DMI resistance in S. homoeocarpa. In brief, overexpression of two ATP-binding cassette (ABC) transporters, ShatrD and ShPDR1, were confirmed to be involved in DMI resistant S. homoeocarpa isolates that exhibited practical field resistance. Furthermore, ShPDR1 played a significant role in effluxing other chemically unrelated fungicides and demonstrated broad substrate specificity (Hulvey et al. 2012; Sang et al. 2015). Also, cytochrome P450 (CYP450) monooxygenases were likely involved in xenobiotic metabolism (Sang et al. unpublished). A fungal-specific transcription factor that concomitantly regulates ABC-transporters and CYP450s for xenobiotic detoxification was further examined in our most recent studies (Sang et al. unpublished). The results of two ABC-transporters are summarized and the novel xenobiotic detoxification regulation in S. homoeocarpa in relation to multidrug resistance are discussed in this contribution.

**MATERIALS AND METHODS**

*S. homoeocarpa* isolates and *in vitro* sensitivity tests to fungicides

The panel of eight isolates consists of 4 DMI insensitive isolates (WBI7, HRI11, SMI27, and HFI40) and 4 DMI sensitive isolates (HRS10, SMS27, JTS30, and HFS35). The isolates were collected from the following sites: four golf courses with previous exposures to fungicides, Hickory Ridge Golf Club (HR), Hartford Golf Club (HF), Wintonbury Hills Golf Club (WB), and Shuttle Meadow Country Club (SM) and one baseline site with no previous exposure, Joseph Troll Turf Research Center (JT) at the University of Massachusetts, Amherst, MA, USA (Popko et al. 2011; Hulvey et al. 2012; Sang et al. 2015).

*In vitro* sensitivity tests of 8 isolates to DMI (propiconazole), dicarboximide (iprodione) and succinate dehydrogenase inhibitor (boscalid) fungicides were conducted by Sang et al. (2015), and EC50 values of propiconazole and iprodione and EC95 values of boscalid were estimated. Agar plugs of 2 day-grown fungal isolates on PDA (potato dextrose agar) were placed on minimal media (MM, 1L; 10 g glucose, 1.5 g K2HPO4, 2 g KH2PO4, 1 g (NH4)2SO4, 0.5 g MgSO4•7H2O, 2 g yeast extract, and 12.5 g agar) unamended and amended with following concentrations of commercial formulations of propiconazole (0.001, 0.01, 0.1, 1, and 10 μg mL−1), iprodione (0.01, 0.1, 1, 10, and 100 μg mL−1) and boscalid (1, 1000, 3000, 5000, and 10000 μg mL−1). One diameter from the agar plug to 8 days-grown colony margin was measured with 16EX digital calipers (Mahr, Göttingen, Germany). EC50 values for propiconazole and iprodione and EC95 values for boscalid were calculated according to modified procedures of Jo et al. (2006). Analysis of variance (ANOVA) and Fisher’s Protected LSD were conducted to compare mean propiconazole and iprodione EC50 values and mean boscalid EC95 values between two groups of DMI sensitive and insensitive isolates.
RNA extraction, cDNA synthesis, and quantitative real-time PCR
The whole process to obtain relative expression values of ShatrD and ShPDR1 in 8 isolates was described in Hulvey et al. (2012) and Sang et al. (2015). In brief, RNA samples were extracted from four DMI sensitive isolates and four DMI insensitive isolates before and after exposure (1 hour) to propiconazole (0.1 μg mL⁻¹). The QuantiTect reverse transcription kit (Qiagen Inc., Valencia, CA, USA) was used for cDNA synthesis with each sample of RNA. The Actin (Shact) gene in S. homoeocarpa was used as a housekeeping gene. Quantitative real-time PCR (qPCR) was performed using Absolute Blue SYBR qPCR MasterMix (Thermo Fisher Scientific, Waltham, MA, USA). Mastercycler ep realplex thermocycler (Eppendorf, Hamburg, Germany) was used for qPCR and the conditions were as follows: one cycle of 15 min at 95°C, and 40 cycles of 15 s at 95°C, 30 s at 60°C and 30 s at 72°C. The relative gene expression value was calculated using comparative C_T method (Livak and Schmittgen, 2001). Analysis of variance (ANOVA) was conducted to compare the mean relative constitutive and induced expression values of ShatrD and ShPDR1 between Groups of DMI sensitive and insensitive isolates (Table 1).

Heterologous expression of ShatrD and ShPDR1 in yeast
Transformants of Saccharomyces cerevisiae hypersensitive strain (AD12345678: AD1-8) transformed with the galactose-inducible expression vector pYES2 with ShPDR1 (AD1-8:PDR1-1 and -2) and with the empty pYES2 vector (AD1-8-pYES2) were generated by Sang et al. (2015). The full-length cDNA sequences of ShatrD were cloned into plasmid pYES2 (Invitrogen, Carlsbad, CA, USA) and the construct was transformed into Escherichia coli DH5α. S. cerevisiae strain AD1-8 (Decottignies et al. 1998) was transformed with the purified vector pYES2 containing ShatrD from E. coli DH5α to generate two independent transformants expressing ShatrD in the presence of galactose (AD1-8:atrD-1 and -2). All transformants were grown at 30°C for 3 days in liquid YNB media lacking uracil and containing 2% galactose. Cell suspensions were diluted to an optical density at 600 nm (OD600) of 0.5 in the liquid media using the VERSAmaxTM microplate reader (Molecular Devices Corporation, Sunnyvale, CA, USA). 5 μL of each yeast transformant were spotted onto YNB agar medium lacking uracil, containing 2% galactose and amended with 0.002 μg mL⁻¹ of propiconazole, 250 μg mL⁻¹ of iprodione and 300 μg mL⁻¹ of boscalid. The sensitivity of yeast transformants to respective fungicides was qualitatively assessed after incubation at 30°C for 3 days. Two biological replicates and three technical replicates per biological replicate were conducted for each transformant and treatment.

RESULTS
Sensitivity of S. homoeocarpa isolates to propiconazole, iprodione, and boscalid
The group of DMI insensitive isolates (DMI I) has significantly higher mean EC₅₀ values of propiconazole (P = 0.0058) and iprodione (P = 0.0162), and mean EC₉₅ values of boscalid (P = 0.0019) than the group of DMI sensitive isolates (DMI S) (Table 1).
Table 1. Mean comparison of in vitro sensitivity to fungicides between two groups of DMI insensitive and sensitive isolates of S. homoeocarpa.

<table>
<thead>
<tr>
<th>Group of Isolates(^a)</th>
<th>Propiconazole EC(_{50}) (μg mL(^{-1}))</th>
<th>Iprodione EC(_{50}) (μg mL(^{-1}))</th>
<th>Boscalid EC(_{95}) (μg mL(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMI insensitive (DMI I)</td>
<td>0.53 a (^b)</td>
<td>0.72 a</td>
<td>6499.3 a</td>
</tr>
<tr>
<td>DMI sensitive (DMI S)</td>
<td>0.03 b</td>
<td>0.50 b</td>
<td>3485.5 b</td>
</tr>
</tbody>
</table>

\(^a\) Field isolates were grouped based on the qualitative growth of isolates at 1 μg mL\(^{-1}\) of propiconazole described in Popko et al. (2012): four DMI insensitive (DMI I) isolates could grow but four DMI sensitive (DMI S) isolates couldn’t.

\(^b\) Mean values followed by the same letter are not significantly different based on Fisher’s protected least significant difference test (α=0.05).

\(^c\) * and ** represent significant difference at \(P < 0.05\) and 0.01, respectively.

Figure 1 Mean relative expression (RE) values for Shatr\(D\) and ShPDR\(1\) in the DMI sensitive (DMI S) and DMI insensitive (DMI I) groups of isolates collected from four exposed sites and one baseline site in New England regions. Gray bars and black bars indicate mean constitutive RE values and mean propiconazole-induced RE values, respectively. Error bars represent 1 standard error from the mean. (A) Constitutive and induced mean RE of Shatr\(D\). (B) Constitutive and induced mean RE of ShPDR\(1\) (adapted from Hulvey et al. 2012; Sang et al. 2015).

Quantitative relative expression of Shatr\(D\) and ShPDR\(1\)
The mean constitutive RE values of Shatr\(D\) and ShPDR\(1\) from the DMI I group were significantly higher than the DMI S group (\(P < 0.0001\)). In response to propiconazole, the DMI I group showed significantly higher mean induced RE values of Shatr\(D\) and ShPDR\(1\) than the DMI S group (\(P < 0.0001\)) (Fig. 1).
Heterologous expression of ShatrD and ShPDR1 in yeast

Transformants of the hypersensitive mutant strain AD12345678 (AD1-8) of *S. cerevisiae* transformed with full-length cDNA of ShPDR1 and ShatrD were designated AD1-8:PDR1-1 and AD1-8:PDR1-2, and AD1-8:atrD-1 and AD1-8:atrD-2, respectively which were able to grow on media amended with propiconazole (0.002 μg mL⁻¹), boscalid (300 μg mL⁻¹) and iprodione (250 μg mL⁻¹), but the yeast mutant containing the empty vector (AD1-8-pYES2) was unable to grow on media amended with aforementioned fungicides (Fig. 2).

![Figure 2](image-url)

**Figure 2**  Effect of heterologous overexpression of two ABC-transporters ShPDR1 or ShatrD from *S. homoeocarpa* in *S. cerevisiae* strain AD12345678 on in vitro sensitivity to propiconazole, boscalid and iprodione. Each column (from left to right) represents one *S. cerevisiae* control transformant with the empty vector pYES2 (AD1-8-pYES2) and two independent *S. cerevisiae* transformants with plasmids containing the full length ShPDR1 (AD1-8:PDR1-1 and -2) (Sang et al. 2015) or ShatrD (AD1-8:atrD-1 and -2).

**DISCUSSION**

The current results adapted from Hulvey et al. (2012) and Sang et al. (2015) indicate that DMI insensitive field isolates exhibit reduced sensitivity to iprodione and boscalid through constitutive and induced overexpression of two MDR ABC transporters, ShatrD and ShPDR1. Furthermore, yeast transformant with heterologous expression of ShatrD and ShPDR1 was capable of effluxing propiconazole, boscalid and iprodione, which led to decreased *in vitro* fungicide sensitivity. These MDR resistance phenotypes caused by overexpression of DMI orazole mediating ABC transporters were well described in other fungal systems such as PDR5 from *S. cerevisiae* and CDR1 from *Candida albicans* (Rogers et al. 2001; Prassad et al. 1995). To expand our knowledge on fungicide resistance beyond the limited results from Hulvey et
Our findings indicate that overexpression of a cytochrome P450 monooxygenase and overexpression of ABC transporters may confer resistance to multiple fungicides by detoxification in some of DMI insensitive *S. homoeocarpa* field populations. In addition, a novel fungal specific-transcription factor may regulate xenobiotic detoxification genes/proteins and its gain-of-function mutation in one of field populations results in constitutive overexpression of xenobiotic detoxification genes (Sang et al. unpublished). This new and improved understanding will further build on the body of knowledge of MDR resistance conferred by xenobiotic detoxification regulation for plant and human pathogenic fungi, and facilitate the discovery of new drug targets to control pathogenic fungal populations.

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