Jung G, et *al.*, Multidrug Resistance Conferred by Xenobiotic Detoxification in the Ascomycete Fungus *Sclerotinia homoeocarpa*. In: Deising HB; Fraaije B; Mehl A; Oerke EC; Sierotzki H; Stammler G (Eds), "Modern Fungicides and Antifungal Compounds", Vol. VIII, pp. 101-106. © 2017 Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-15-0

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

Jung G[1], Sang H[1], Hulvey J[2], Chang T[3], Popko J[1]

[1]*Uni. of Massachusetts, Stockbridge School of Agriculture, 161 Holdsworth Ways, Amherst, MA 01003, USA*

[2]*Uni. of Massachusetts, Biology Depart., 611 North Pleasant Str, Amherst, MA 01003, USA*

[3]*Kyungpook National Univ., School of Ecology & Environmental System, Sangju, South Korea*

Email: jung@umass.edu

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

Persistent Identifier: urn:nbn:de: 0294-sp-2017-Reinh-13-6

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 EC_{50} values of propiconazole and iprodione and EC_{95} 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 K_2HPO_4, 2 g KH_2PO_4, 1 g $(NH_4)_2SO_4$, 0.5 g $MgSO_4 \bullet 7H_2O$, 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). EC_{50} values for propiconazole and iprodione and EC_{95} 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 EC_{50} values and mean boscalid EC_{95} 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 galatose-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 galatose (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 (OD_{600}) 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 EC_{50} values of propiconazole ($P = 0.0058$) and iprodione ($P = 0.0162$), and mean EC_{95} values of boscalid ($P = 0.0019$) than the group of DMI sensitive isolates (DMI S) (Table 1).

Persistent Identifier: urn:nbn:de: 0294-sp-2017-Reinh-13-6

Table 1. Mean comparison of *in vitro* sensitivity to fungicides between two groups of DMI insensitive and sensitive isolates of *S. homoeocarpa*.

Group of Isolates[a]	Propiconazole EC_{50} (µg mL^{-1})	Iprodione EC_{50} (µg mL^{-1})	Boscalid EC_{95} (µg mL^{-1})
DMI insensitive (DMI I)	0.53 a[b]	0.72 a	6499.3 a
DMI sensitive (DMI S)	0.03 b	0.50 b	3485.5 b
P value[c]	**	*	**

[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.

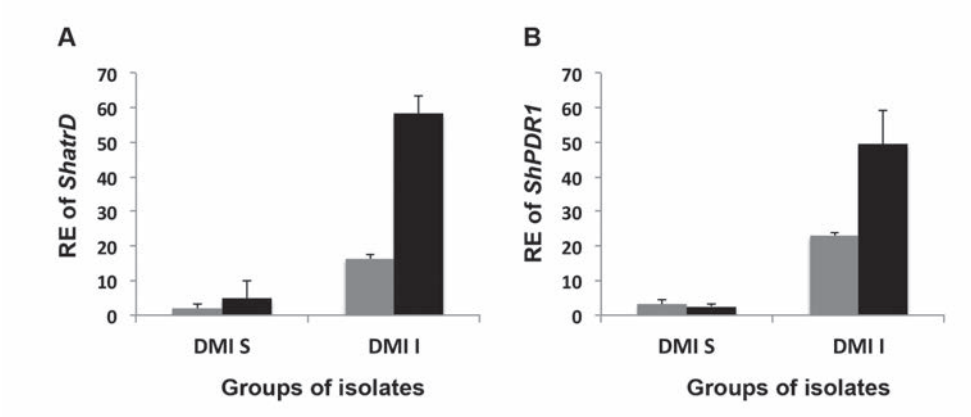

Figure 1 Mean relative expression (RE) values for *ShatrD* and *ShPDR1* in the DMI sensitive (DMI S) and DMI insensitive (DMI I) groups of isolates collected from four exposed sites and one baseline site in New England regions. Gray bars and black bars indicate mean constitutive RE values and mean propiconazole-induced RE values, respectively. Error bars represent 1 standard error from the mean. (A) Constitutive and induced mean RE of *ShatrD*. (B) Constitutive and induced mean RE of *ShPDR1* (adapted from Hulvey et *al.* 2012; Sang et *al.* 2015).

Quantitative relative expression of ShatrD and ShPDR1

The mean constitutive RE values of *ShatrD* and *ShPDR1* 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 *ShatrD* and *ShPDR1* than the DMI S group ($P < 0.0001$) (Fig. 1).

Heterologous expression of ShatrD and ShPDR1 in yeast

Transforments 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).

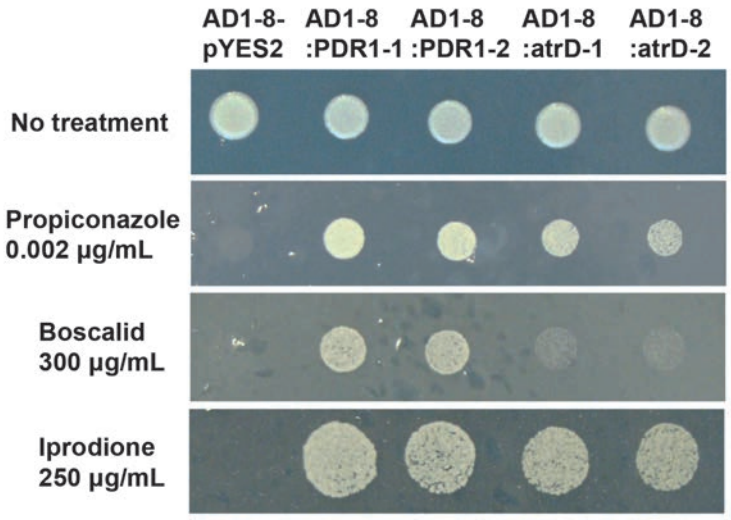

Figure 2 Effect of heterologous overexpression of two ABC-transporters *ShPDR1* or *ShatrD* from *S. homoeocarpa* in *S. cerevisiae* strain AD12345678 on *in vitro* sensitivity to propiconazole, boscalid and iprodione . Each column (from left to right) represents one *S. cerevisiae* control transformant 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 or azole 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

al. (2012) and Sang et *al.* (2015), we further investigated a xenobiotic detoxification pathway using the transcriptomic data and the genetic transformation system in *S. homoeocarpa*.

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.

REFERENCES

Decottignies A; Grant A; Nichols J; de Wet H; McIntosh, D.; Goffeau A (1998). ATPase and multidrug transport activities of the overexpressed yeast ABC protein Yor1p. *Journal of Biological Chemistry* 273, 12612-12622.

Detweiler AR; Vargas JMJ; Danneberger TK (1983). Resistance of *Sclerotinia homoeocarpa* to iprodione and benomyl. *Plant Disease* 67, 627-630.

Hulvey J; Popko JT; Sang H; Berg A; Jung G (2012). Overexpression of ShCYP51B and ShatrD in *Sclerotinia homoeocarpa* isolates exhibiting practical field resistance to a demethylation inhibitor fungicide. *Appl Environ Microbiol* 78, 6674-6682.

Jo Y; Niver A; Rimelspach J; Boehm MJ (2006). Fungicide sensitivity of *Sclerotinia homoeocarpa* from golf courses in Ohio. *Plant Disease* 90, 807-813.

Livak K; Schmittgen T (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)). *Methods* 25, 402-408.

Popko JT; Ok C; Campbell-Nelson K; Jung G (2012). The association between in vitro propiconazole sensitivity and field efficacy of five New England *Sclerotinia homoeocarpa* populations. *Plant Disease* 96, 552-561.

Prasad R; De Wergifosse P; Goffeau A; Balzi E (1995). Molecular cloning and characterization of a novel gene of Candida albicans, CDR1, conferring multiple resistance to drugs and antifungals. *Current Genetics* 27, 320-329.

Rogers B; Decottignies A; Kolaczkowski M; Carvajal E; Balzi E; Goffeau A (2001). The pleitropic drug ABC transporters from *Saccharomyces cerevisiae*. *Journal of Molecular Microbiology and Biotechnology* 3, 207-214.

Sang H; Hulvey J; Popko JT; Lopes J; Swaminathan A; Chang T; Jung G (2015). A pleiotropic drug resistance transporter is involved in reduced sensitivity to multiple fungicide classes in *Sclerotinia homoeocarpa*. *Molecular Plant Pathology* 16, 251-261.

Vargas JMJ; Golembiewski R; Detweiler AR (1992). Dollar spot resistance to DMI fungicides. *Golf Course Management* 60, 50-54.