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Monitoring and analysis of rice pathogen *Ustilaginoidea virens* isolates with resistance to sterol demethylation inhibitors in China

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Abstract

Rice false smut (RFS), caused by *Ustilaginoidea virens* (Cooke) Takah, is an important fungal disease of rice. In China, sterol demethylation inhibitors (DMIs) are common fungicides used to control RFS. In a previous study, we detected two propiconazole-resistant *U. virens* isolates in 2015 in Huai'an city, Jiangsu Province, China. In the current study, we detected six propiconazole-resistant isolates out of 180 *U. virens* isolates collected from rice fields in Jiangsu Province in 2017, and found they were from three different places (Xuzhou, Huai'an and Jintan). All these six propiconazole-resistant isolates were cross-resistant to three other sterol demethylation inhibitor (DMI) fungicides, i.e. difenoconazole, tebuconazole, and epoxiconazole. Among them, two isolates (2017–61 and 2017–170) had high fitness. Through sequencing and RT-qPCR analysis, we found that the expression levels of *CYP51* and its encoded protein were significantly increased in the propiconazole-resistant isolates with a "CC" insertion mutation upstream of the *CYP51* coding region compared to the propiconazole-sensitive isolates. In addition, propiconazole stimulated *CYP51* expression in all isolates. Propiconazole also stimulated the accumulation of CYP51 protein in propiconazole-sensitive isolates and propiconazole-resistant isolates without mutation, but not in propiconazole-resistant isolates with the "CC" mutation. According to JASPAR database analysis, the predicated functional binding sites for propiconazole-resistant isolates with a "CC" insertion mutation and propiconazole-sensitive isolates were different. Given the high fitness of the propiconazole-resistant isolates, the development of resistance to DMIs in *U. virens* should be monitored. Furthermore, we speculated that the over-expression of *CYP51* may contribute to DMI resistance in *U. virens* with the "CC" insertion mutation.

Keywords: *Ustilaginoidea virens*, Propiconazole, Resistance monitoring, Fitness

Background

Rice false smut (RFS), caused by *Ustilaginoidea virens* (Cooke) Takah, is a major fungal disease in most rice-growing areas all over the world. The typical symptom of this disease is the formation of false smut balls in rice grains, which affects the overall quality of rice (Zhou et al. 2008). *U. virens* also produces toxic secondary metabolites, including ustilaginoidins and ustiloxins, which threaten food and feed safety (Qiu et al. 2019). Currently, the application of fungicides is a fast and effective way to control this disease. Sterol demethylation inhibitors (DMIs), mainly including propiconazole, difenoconazole and tebuconazole, have been most widely used to control RFS in China (Chen et al. 2013). However, due to the intensive application of DMI fungicides, resistance has been detected in several important pathogens, such as *Monilinia fructicola*, *Puccinia triticina*, *Penicillium digitatum*, and *Erysiphe graminis*, in recent years (Schepers 1985; Delye et al. 1998; Luo and Schnabel 2008; Price et al. 2015). In 2015, Zhou et al. (2019) reported propiconazole-resistant isolates of *U. virens* and found one with high fitness. Therefore, monitoring propiconazole resistance in the field and evaluating its resistance risk

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are particularly important for the management of DMI resistance in RFS.

DMIs are broad-spectrum fungicides that inhibit ergosterol biosynthesis by targeting sterol 14α-demethylase CYP51, a key enzyme in sterol biosynthesis. Homologous genes of *CYP51* have been found in phytopathogenic fungi *Fusarium graminearum*, *Magnaporthe oryzae*, and *Aspergillus* spp. (Mellado et al. 2001; Yin et al. 2009; Yan et al. 2011). However, only one *CYP51* was identified in the *U. virens* genome (Wang et al. 2015). The common mechanisms of fungal resistance to DMI include mutations in the target *CYP51* gene; overexpression of the *CYP51* gene, and enhanced efflux pumps that are encoded by ATP-binding cassette (ABC) transporter (Ma et al. 2006; Kretschmer et al. 2009; Cools et al. 2010, 2012; Wang et al. 2015; Tsao et al. 2016). In *F. graminearum*, resistance to DMIs was not only related to the mutations of *CYP51A*, but also related to the ABC transporter (Ammar et al. 2013; Duan et al. 2018). In *M. fructicola*, a transposable element 'Mona' was inserted in the 113 bp upstream of *MfCYP51*, which determined the resistance to DMI fungicides (Luo et al. 2008; Chen et al. 2017). In *U. virens*, up-regulation of *CYP51* and increased ergosterol biosynthesis were reported to be associated with propiconazole resistance (Zhou et al. 2019). However, due to the lack of resistant field isolates, the resistance mechanism of *U. virens* to DMI fungicides requires further investigation.

Thus, the major objectives of the study are to (i) monitor the resistance of *U. virens* to propiconazole in Jiangsu Province of China, (ii) assess the resistance risk of *U. virens* to DMI fungicides, and (iii) investigate the possible mechanisms of DMI fungicide resistance in *U. virens*.

Results

Frequency and distribution of propiconazole-resistant isolates of *U. virens* at the sampling regions

A total of 180 field isolates of *U. virens* were used to monitor the resistance of this pathogen to propiconazole, and six of them (2017–2, 2017–6, 2017–61, 2017–170, 2017–176, and 2017–179) could grow on PSA plates containing 1 μg/mL of propiconazole. As shown in Table 1, among these six propiconazole-resistant isolates, two (2017–2 and 2017–6) were isolated from Xuzhou; one (2017–61) was from Huai'an; and three (2017–170, 2017–176 and 2017–179) were from Jintan. The resistance frequency of *U. virens* to propiconazole in these places was 6.7, 3.0, and 10.3%, respectively.

Sensitivity test of the preliminarily screened resistant isolates of *U. virens* to propiconazole

As shown in Fig. 1, the propiconazole-sensitive isolates 2017–11 and 2017–48 could not grow on PSA plates containing 0.4 μg/mL of propiconazole. However, the six aforementioned propiconazole-resistant isolates could grow on PSA plates with 10 μg/mL of propiconazole. The propiconazole sensitivity (EC_{90}) of these resistant isolates significantly increased compared to that of the sensitive isolate 2017–11. All these resistant isolates were lowly resistant to propiconazole with resistance factor (RF) values less than 10.0 (Table 2).

Cross-resistance analysis of propiconazole-resistant *U. virens* isolates to other DMIs

Based on the EC_{50} values, the isolates 2017–11 and 2017–48 that were sensitive to propiconazole were also sensitive to epoxiconazole, difenoconazole, and tebuconazole (Table 3). In contrast, all six propiconazole-resistant isolates were resistant to these three tested DMIs with resistance factors (RF) ranging from 2.2 to 4.6, 2.0 to 6.0, and 1.7 to 3.7, respectively. It was worth noting that the RF values of 2017–6 to difenoconazole and tebuconazole, and 2017–170 to tebuconazole were less than 1, but the EC_{90} values of them were much higher than that of the sensitive isolates (Table 3).

Mycelial growth, sporulation and pathogenicity of propiconazole-resistant *U. virens* isolates

The mycelial growth of all six propiconazole-resistant isolates was slower than that of the propiconazole-sensitive isolate 2017–11. However, there was no significant difference in mycelial growth between resistant isolates and the sensitive isolate 2017–48, and the mycelial

Table 1 Frequency and distribution of resistance of *Ustilaginoidea virens* to propiconazole in 2017

Sampling place		Number of isolates tested	Number of resistant isolates	Frequency of resistance (%)
Jiangsu	Xuzhou	30	2 (2017–2, 2017–6)	6.7
	Huai'an	33	1 (2017–61)	3.0
	Jintan	29	3 (2017–170, 2017–176, 2017–179)	10.3
	Nantong	12	0	0
	Yangzhou	30	0	0
	Nanjing	22	0	0
	Ganyu	16	0	0
	Yancheng	7	0	0

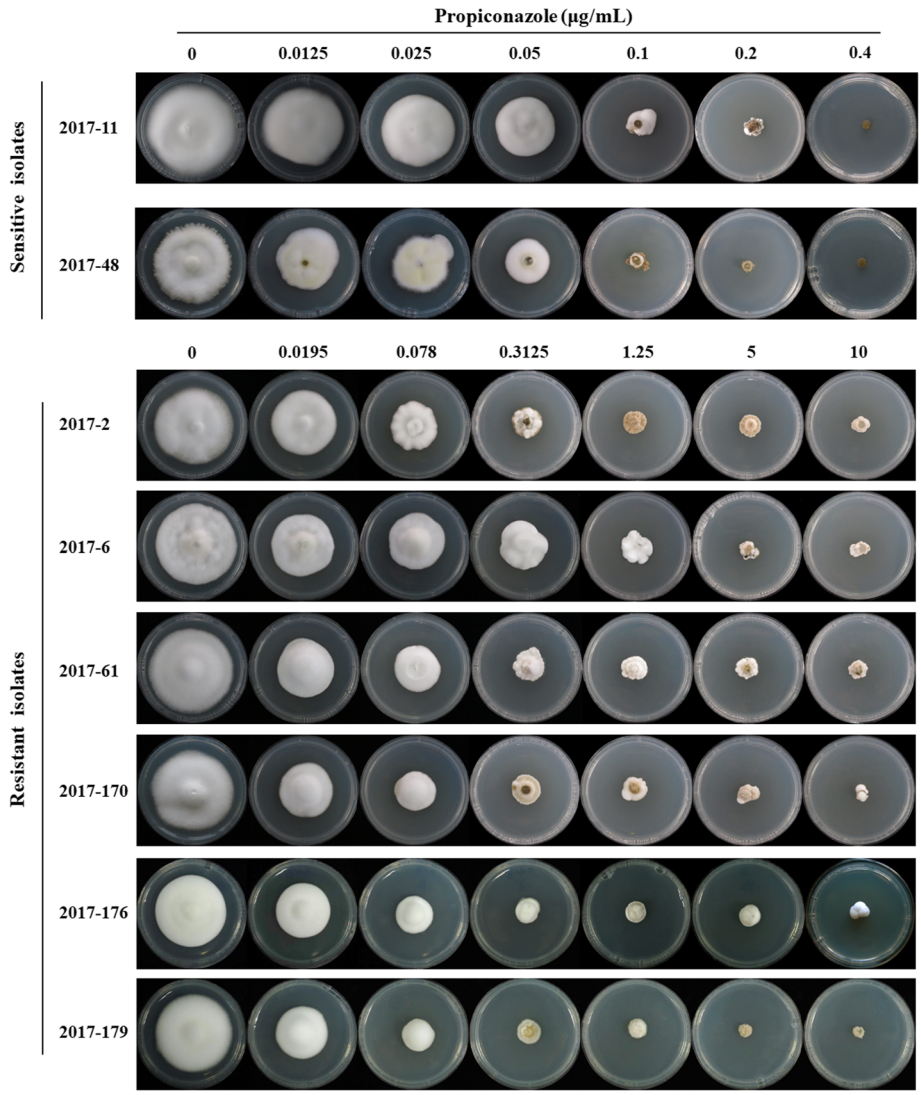

Fig. 1 Comparison of propiconazole sensitivity between propiconazole-sensitive and propiconazole-resistant *U. virens* isolates

Table 2 Sensitivity of the preliminarily screened resistant isolates of *Ustilaginoidea virens* to propiconazole

Isolates	Origin	EC_{50} (μg/mL)	EC_{90} (μg/mL)	RF	Resistance level
2017–11	Sensitive	0.053	0.26	/	
2017–48	Sensitive	0.034	0.19	/	
2017–2	Resistant	0.13	10.70	2.6	LM
2017–6	Resistant	0.24	21.03	4.8	LM
2017–61	Resistant	0.081	22.03	1.6	LM
2017–170	Resistant	0.058	12.09	1.2	LM
2017–176	Resistant	0.12	12.80	2.4	LM
2017–179	Resistant	0.048	3.18	1.0	/

Table 3 Cross-resistance of propiconazole-resistant isolates of *Ustilaginoidea virens* to other DMIs

Isolates	Epoxiconazole				Difenoconazole				Tebuconazole			
	EC_{50} (μg/mL)	EC_{90} (μg/mL)	RF	Resistance level	EC_{50} (μg/mL)	EC_{90} (μg/mL)	RF	Resistance level	EC_{50} (μg/mL)	EC_{90} (μg/mL)	RF	Resistance level
2017–11	0.037	0.18	/		0.066	0.33	/		0.11	0.47	/	
2017–48	0.039	0.14	/		0.11	0.70	/		0.068	0.25	/	
2017–2	0.17	3.68	4.6	LR	0.38	11.56	4.3	LR	0.33	8.23	3.7	LR
2017–6	0.099	1.86	2.7	LR	0.048	51.76	0.5	/	0.048	11.70	0.5	/
2017–61	0.10	1.01	2.7	LR	0.29	2.50	3.3	LR	0.19	1.45	2.1	LR
2017–170	0.083	2.76	2.2	LR	0.23	6.06	2.6	LR	0.083	15.33	0.9	/
2017–176	0.12	5.20	3.2	LR	0.53	12.28	6.0	LR	0.15	12.42	1.7	LR
2017–179	0.11	6.45	3.0	LR	0.18	74.42	2.0	LR	0.20	25.54	2.2	LR

growth of several resistant isolates was even faster than that of 2017–48 (Table 4). Two resistant isolates, 2017–61 and 2017–170, exhibited increased spore production in potato sucrose broth (PSB) in comparison to the propiconazole-sensitive isolate 2017–11. In addition, the virulence of the field-resistant isolate 2017–61 was stronger than that of the sensitive isolates 2017–11 and 2017–48 (Table 4).

Sequence and expression analysis of the *CYP51* and its encoded protein in propiconazole-resistant *U. virens* isolates

A multisequence alignment showed no mutations in the coding region of *CYP51*, a reported target gene of DMIs, for all six propiconazole-resistant isolates (data not shown). However, an insertion of two bases (CC) was found 154-bp upstream of the *CYP51* coding region in 2017–61, 2017–170 and 2017–179 (Fig. 2a). The GenBank accession numbers were MT701730, MT701731, and MT701732 for 2017-61, 2017-170, and 2017-179, respectively. The expression levels of *CYP51* and its encoded protein were significantly increased in these three resistant isolates compared to the sensitive isolate 2017–11 (Fig. 2b, c). In addition, the expression of *CYP51* was significantly up-regulated by propiconazole in all isolates. However, the protein accumulation of CYP51 was increased in sensitive isolate and also in resistant isolates without the 'CC' mutation treated with 1 μg/mL of propiconazole, but not in propiconazole-resistant isolates with the "CC" mutation (Fig. 2c).

Prediction of functional binding sites in the promoter of *CYP51*

The promoter region predication of *CYP51* in *U. virens* was made using the online BDGP database. In the promoter region, 118 and 132 putative transcription factor binding sites were predicted in sequence A (without the 'CC' insertion mutation) and B (with the 'CC' insertion mutation) using JASPAR online software, respectively (Additional file 1: Tables S1 and S2). There were 20 different putative binding sites between sequences A and B. Among them, 16 binding sites were specific to sequence B with 'CC' insertion mutations, and the binding abilities of two sites of sequence B were greater than that of sequence A (Table 5).

Discussion

DMI fungicides such as Armure (30% difenoconazole-propiconazole) and Horizon (tebuconazole) have been

Table 4 Mycelial growth, sporulation and virulence of propiconazole-resistant isolates

Isolates	Mycelial growth	Spore production	Virulence
	Colony diameter (cm)	Conidium in vitro ($\times 10^5$/mL)	Number of false smut balls per rice panicle
2017–11	45.8 ± 0.8 e	9.6	2.0
2017–48	42.6 ± 0.8 b	11.8	2.9
2017–2	43.4 ± 0.6 bc	6.3	0.1
2017–6	37.5 ± 0.7 a	7.4	0.3
2017–61	41.0 ± 0.8 b	15.3	8.7
2017–170	42.0 ± 0.4 b	10.9	1.4
2017–176	45.0 ± 1.7 d	1.8	0
2017–179	42.2 ± 0.6 b	2.9	0.8

Values followed by different letters are significantly different at $P < 0.05$

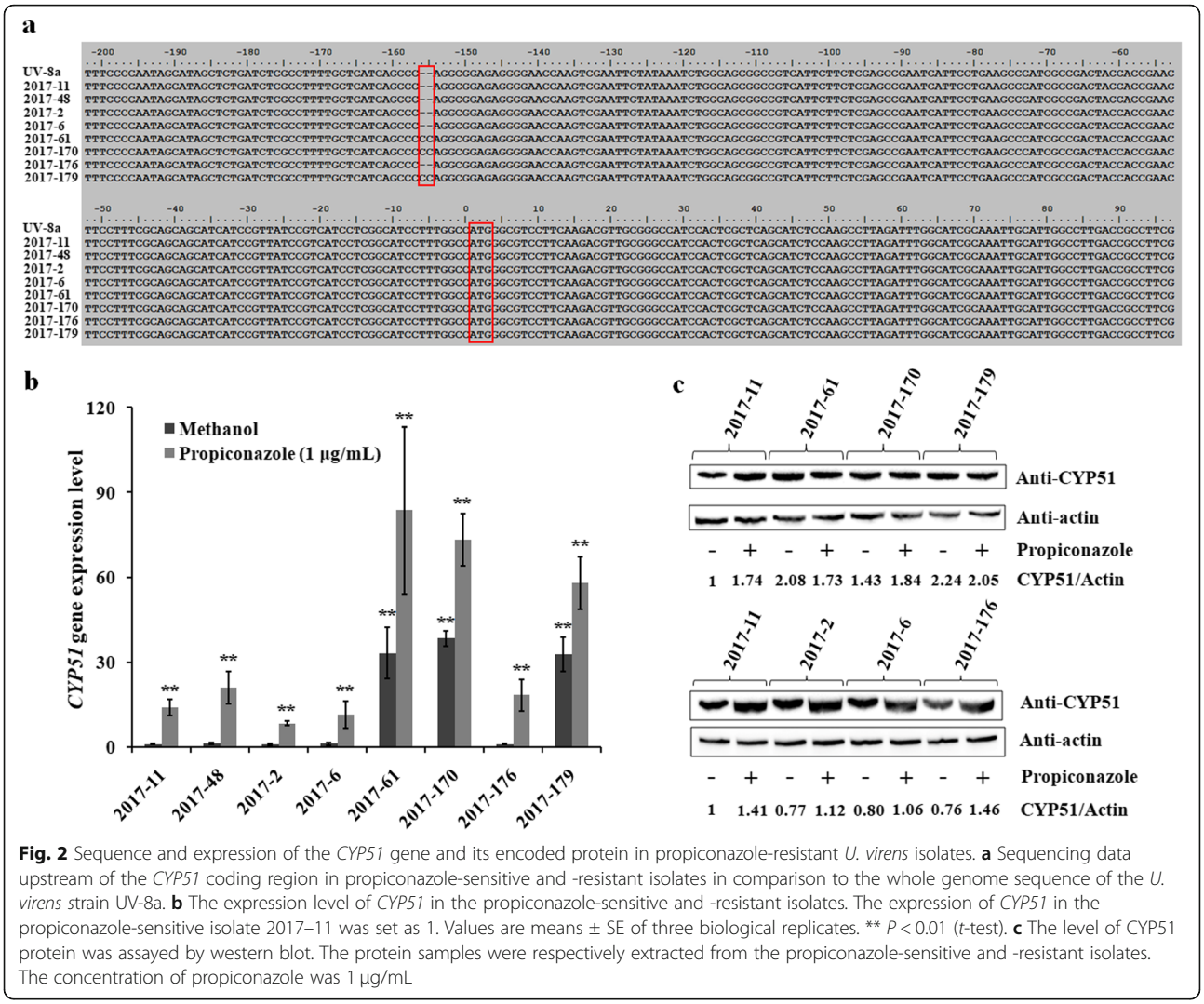

Fig. 2 Sequence and expression of the *CYP51* gene and its encoded protein in propiconazole-resistant *U. virens* isolates. **a** Sequencing data upstream of the *CYP51* coding region in propiconazole-sensitive and -resistant isolates in comparison to the whole genome sequence of the *U. virens* strain UV-8a. **b** The expression level of *CYP51* in the propiconazole-sensitive and -resistant isolates. The expression of *CYP51* in the propiconazole-sensitive isolate 2017–11 was set as 1. Values are means ± SE of three biological replicates. ** $P < 0.01$ (*t*-test). **c** The level of CYP51 protein was assayed by western blot. The protein samples were respectively extracted from the propiconazole-sensitive and -resistant isolates. The concentration of propiconazole was 1 μg/mL

widely used for controlling RFS in the past two decades in China. Wang et al. (2015) obtained DMI-resistant mutants of *U. virens* by UV-irradiation in the laboratory. However, two propiconazole-resistant *U. virens* isolates were detected in Huai'an, Jiangsu Province, in 2015 (Zhou et al. 2019). In the current study, six out of 180 *U. virens* isolates were found to be resistant to propiconazole. In addition to Huai'an, resistant isolates were also detected in Xuzhou and Jintan in 2017. The frequency of resistant isolates in Huai'an, Xuzhou, and Jintan were 3.0, 6.7 and 10.3%, respectively (Table 1). These results indicated that the resistance of *U. virens* to DMIs evidently increased from 2015 to 2017.

After isolating the six propiconazole-resistant isolates, we determined their resistance levels and investigated the influence of DMI fungicide resistance on their fitness. We found that the sensitive isolates couldn't grow on PSA plate with 0.4 μg/mL of propiconazole, but the resistant isolates could grow on PSA plate containing 10 μg/mL of propiconazole (Fig. 1). The RF values (Table 2) of these resistant isolates further demonstrated that the propiconazole-resistance levels of *U. virens* were low. In addition, the propiconazole-resistant isolates 2017–61 and 2017–170 did not reduce in mycelia growth, sporulation and pathogenicity compared to those of propiconazole-sensitive isolates, which indicated that their fitness values were high (Table 4). The high fitness of propiconazole-resistant isolates suggested that the resistance of *U. virens* to DMI fungicides may continue to develop because of the strong competitiveness of resistant populations. Moreover, there was positive cross-resistance between propiconazole and other DMI fungicides (Table 3), indicating that the resistant isolates collected in the field were not selectively resistant to DMI fungicides at present.

As noted above, some pathogens are resistant to DMI fungicides. In most cases, resistance is linked to point

Table 5 Differential functional binding sites predicted in the non-coding region upstream of *CYP51* in *U. virens* with 'CC' insertion mutations (sequence B) relative to that without 'CC' insertion (sequence A) using the JASPAR database

Model ID	Model name	Score		Predicted proteins in *U. virens*	Percent identity (%)	Annotation
		Sequence A	Sequence B			
MA0351.1	DOT6	9.81	10.63	KDB15421.1	45.71	MYB DNA-binding domain-containing protein
MA0350.1	TOD6	10.32	8.76	KDB15421.1	51.43	MYB DNA-binding domain-containing protein
MA0316.1	HAP5	5.51	9.05	KDB16258.1	79.71	CCAAT-binding protein subunit HAP5
MA0285.1	CRZ1	/	8.99	KDB16057.1	64.10	C2H2 type zinc finger domain-containing protein
MA0313.1	HAP2	2.36	/	KDB14896.1	69.86	CCAAT-binding complex subunit HAP2
MA0394.1	STP1	/	3.44	/	/	/
MA0268.1	ADR1	/	5.25	KDB12570.1	36.65	DNA binding regulatory protein AmdX
MA0268.1	ADR1	/	6.89	KDB12570.1	36.65	DNA binding regulatory protein AmdX
MA0337.1	MIG1	/	9.07	KDB11811.1	72.86	Carbon response regulator
MA0341.1	MSN2	/	7.93	KDB13421.1	58.33	Cutinase G-box binding protein
MA0342.1	MSN4	/	7.02	KDB13421.1	54.67	Cutinase G-box binding protein
MA0362.1	RDS2	/	5.91	KDB19075.1	32.41	Putative Zn cluster transcription factor Rds2
MA0276.1	ASH1	/	4.71	/	/	/
MA0332.1	MET28	/	5.71	KDB18401.1	36.51	Regulatory protein cys-3
MA0338.1	MIG2	/	7.14	/	/	/
MA0339.1	MIG3	/	7.53	KDB11811.1	61.29	Carbon response regulator
MA0341.1	MSN2	/	4.15	KDB13421.1	58.33	Cutinase G-box binding protein
MA0342.1	MSN4	/	4.13	KDB13421.1	54.67	Cutinase G-box binding protein
MA0364.1	REI1	/	5.71	KDB10897.1	33.55	C2H2 type zinc finger containing protein
MA0436.1	YPR022C	/	5.83	KDB11753.1	44.07	Zinc finger protein odd-paired-like

mutations in the target gene *CYP51* (De Waard 1996; Marichal et al. 1999; Wyand and Brown 2005; Cools and Fraaije 2013). The Y137H mutation in CYP51 protein in the resistant mutant of *U. virens*, previously reported to be generated by UV irradiation, conferred resistance to tebuconazole. In addition, overexpression of *CYP51* was observed in the tebuconazole-resistant mutant (Wang et al. 2015). However, in the current study, no mutations in the *CYP51* gene were detected in any of the six propiconazole-resistant isolates, which was consistent with the resistant isolates of *U. virens* screened in 2015 (Zhou et al. 2019).

In *M. fructicola*, a 65-bp sequence inserted at 113 bp upstream of the *MfCYP51* gene was reported to be associated with DMI fungicide resistance (Chen et al. 2017). In this study, the 'CC' insertion was found in three resistant isolates (2017–61, 2017–170, and 2017–179) at 154 bp upstream of the *CYP51* gene as previously reported in resistant isolate 88 collected in 2015 (Zhou et al. 2019). In addition, the expression levels of *CYP51* and its encoded protein were significantly increased in these isolates (Fig. 2). The results suggested that 'CC' insertion mutation may influence the expression of *CYP51* by altering the ability of transcription factors to participate in regulation, which may reduce sensitivity of *U. virens* to DMI fungicides. In support of this inference, we used JASPAR (the largest transcription-factor binding profile database) to predict the functional binding sites of sequences with or without 'CC' insertion mutation (Wasserman and Sandelin 2004; Mathelier et al. 2014). As expected, 16 additional functional binding sites were found to be specific to the sequence with the 'CC' insertion mutation rather than to that without the 'CC' insertion mutation, and the binding abilities of two sites of sequence B were stronger than that of sequence A (Table 5).

In addition, most of the predicted transcription factors were involved in cell wall integrity or osmotic stress. For example, the Myb-like HTH transcription factor Dot6 in *Candida albicans* was reported to be involved in the TOR signaling pathway (Chaillot et al. 2019), which was regulated in the responses to cell wall damaging stress in *F. graminearum* (Gu et al. 2015). Hap2/5, CRZ1, ADR1, MIG1/2/3, MSN2/4, and RDS2 have been reported to function directly in cell wall integrity or osmotic stress (Proft and Serrano 1999; Mendizabal et al. 2001; Wong et al. 2003; Panadero et al. 2007; Moreno et al. 2008; Nino-Vega et al. 2009; Li et al. 2013; Liu et al. 2013; Thewes 2014; Manzanares-Estreder et al. 2017; Jung et al. 2018; Miller et al. 2019). Combined with previous

studies showing that the *CYP51* gene encodes an enzyme that is part of the cell membrane, the current predictions further suggested that the resistance of *U. virens* to DMI fungicides may be related to *CYP51* overexpression mediated by 'CC' insertion mutations.

In our previous study, propiconazole stimulated the expression of *CYP51* in both propiconazole-sensitive and -resistant isolates (Zhou et al. 2019). The same results were observed in this study (Fig. 2b). However, the level of *CYP51*-encoded protein was increased in propiconazole-sensitive isolates and in propiconazole-resistant isolates that had no mutation with propiconazole treatment, whereas was unchanged in propiconazole-resistant isolates with the 'CC' insertion mutation (Fig. 2c). This result suggested that the DMI resistance may be induced by fungicides in the propiconazole-resistant isolates without any mutations. The underlying resistance mechanisms, for example, whether the ABC transporter is involved in these isolates, need to be further explored.

Conclusions

In this study, we showed a potential risk of *U. virens* resistance to DMI fungicides. Propiconazole-resistant isolates were found in three cities in Jiangsu Province. And all of them were positively cross-resistant to epoxiconazole, difenoconazole, and tebuconazole. In addition, two of them had high fitness. We suggest that it should be necessary to persistently monitor the resistance of *U. virens* to DMIs. DMIs should be valuable when used in alternation with other fungicides for the control of RFS in rice fields.

Methods

Isolates of *U. virens* and fungicides

U. virens isolates 2017–11, 2017–48, 2017–2, 2017–6, 2017–61, 2017–170, 2017–176, and 2017–179 were isolated from eight rice fields in Jiangsu Province of China in 2017 by single spore separation. All the isolates were stored in the rice diseases laboratory, Jiangsu Academy of Agricultural Science, China. Technical-grade propiconazole (96.3%), epoxiconazole (96%), difenoconazole (96%), and tebuconazole (97.3%) were provided by Jiangsu Fengdeng Pesticide Co., Ltd., Jiangsu Feixiang Chemical Co., Ltd., ZIBO DEZUN Chemical Co., and Beijing HWRK Chem Co., Ltd., respectively. All the fungicides were dissolved in methanol at 10 mg/mL and stored at 4 °C in the dark before further use.

In vitro screening of propiconazole-resistant isolates

In order to obtain propiconazole-resistant isolates, mycelia plugs (5 mm in diameter) of 14-day-old isolates were transferred to PSA (potato sucrose agar: 200 g/L of potato, 20 g/L of sucrose, and 15 g/L of agar) plates containing 0.5 μg/mL propiconazole and then transferred to PSA plates with 1 μg/mL propiconazole and incubated at 28 °C for 14 days. The isolates that grew on the PSA plates containing 1 μg/mL propiconazole were propiconazole-resistant isolates. EC_{50} and EC_{90} represented the concentration of propiconazole that resulted in 50 and 90% inhibition of *U. virens*. The average EC_{50} value for the sensitive isolates was 0.05 μg/mL as Zhou et al. (2017) reported. The resistance factor (RF) in this study was the ratio of EC_{50} value of resistant isolates to average EC_{50} value (0.05 μg/mL). RF values less than 10, between 10 and 100, and more than 100 were classified as low-resistance (LR), moderate-resistance (MR), and high-resistance (HR) strains, respectively.

Cross-resistance analysis

Cross resistance between propiconazole and other DMIs (epoxiconazole, difenoconazole, and tebuconazole) in *U. virens* was tested as previously described (Zhou et al. 2019). Mycelia plugs (5 mm in diameter) of each tested isolate were transferred to PSA plates containing different concentration of fungicides at 28 °C for 16 days. For the propiconazole-sensitive isolates, the concentrations of propiconazole, epoxiconazole, difenoconazole, and tebuconazole were 0, 0.0125, 0.025, 0.05, 0.1, 0.2, and 0.4 μg/mL. For the propiconazole-resistant isolates, the concentrations of these fungicides were 0, 0.0195, 0.078, 0.3125, 1.25, 5, and 10 μg/mL. The 50 and 90% effective concentrations (EC_{50} and EC_{90}, respectively) of *U. virens* were calculated as described previously (Duan et al. 2018). There were three replicates of each concentration for each isolate. The experiment was repeated twice.

Mycelial growth, sporulation and pathogenicity

In order to test mycelial growth, mycelia plugs (5 mm in diameter) of 14-day-old isolates were transferred to PSA plates, and then the diameter of the colony was measured after incubation at 28 °C for 18 days. In order to test sporulation, all tested isolates were cultured on PSA plates at 28 °C for 14 days. Then two 5 mm-diameter mycelia plugs were transferred into 100 mL of PSB, and the conidium was counted with a hemocytometer after shaking at 28 °C, 150 rpm for 7 days. In order to test virulence, the conidium and hyphal suspension was injected into rice panicles of Liangyoupeijiu as described by Yu et al. (2015). Each isolate was repeated three times and each experiment was conducted twice.

Sequence analysis of the *CYP51* gene

Genomic DNA was extracted from the mycelia of all tested isolates using a DNA extraction kit (PD biotech, China). The primer pair Uv-CYP51-F/R listed in Additional file 1: Table S3 was designed to amplify the sequence of the *CYP51* gene and its upstream region. PCR

Table 6 Nucleotide sequences used to predict functional binding sites in non-coding region upstream of *CYP51* in *Ustilaginoidea virens*

Name	Nucleotide sequences
sequence A	gatctcgccttttgctcatcagcccaggcggagaggggaacca**agtcgaattgtataaatctggcagcggccgtcattcttctcgagccgaat**
sequence B	gatctcgccttttgctcatcagcccCCaggcggagaggggaaccaagtcgaattgtataaatctggcagcggccgtcattcttctcgagccgaat

The nucleotide sequence in bold font is the predicted promoter region, and the uppercase letters indicate the insertion mutation in propiconazole-resistant isolates

amplifications were performed as described previously (Zhou et al. 2019). The enzyme used in PCR reactions was Phanta Max Super-Fidelity DNA Polymerase (Vazyme, China). The PCR products were purified using Fastpure gel DNA extraction mini kit (Vazyme, China) and sequenced by Tsingke Biological Technology.

RT-qPCR and western blot assays

All the tested isolates were cultured in PSB liquid medium at 28 °C for 4 days, and then treated with 1 μg/mL of propiconazole for 24 h. Mycelia was harvested and washed with sterile water for total RNA and protein extraction. Total RNA and RT-qPCR were performed as described previously (Zhou et al. 2019). Briefly, RNA from mycelia of each isolate was extracted using a Total RNA Rapid Extraction Kit (BioTeke Co., Beijing, China), and reverse transcribed into cDNA using HiScript II 1st Strand cDNA Synthesis Kit (+gDNA wiper) (Vazyme Co., Nanjing, China) according to the manufacturer's instructions. RT-PCR was performed using primers listed in Additional file 1: Table S3. α-tubulin was used as a reference gene. Each isolate was repeated three times. For protein extraction, 50 mg of liquid nitrogen grinded mycelia was re-suspended in 500 μL of protein extraction buffer [50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 5 mM ethylene diamine tetraacetic acid (EDTA), 1% Triton X-100, 2 mM phenylmethylsulfonyl fluoride (PMSF)] supplemented with protease inhibitor cocktail (Beyotime, Shanghai, China). Each sample was loaded onto 12% SDS-PAGE gels. Anti-CYP51A1/CYP51 antibody (Abcam, Cambridge, England) and anti-beta actin antibody (Zoonbio, Nanjing, China) were used at 1:1000 for immunoblot analyses. After inoculation with secondary antibody anti-rabbit IgG-HRP (1:20,000) or anti-mouse IgG-HRP (1:20,000), chemiluminescence was detected as described previously (Li et al. 2019). In brief, the blots were determined by using an ECL substrate kit (Thermo Scientific, USA) and Tanon 5200 automatic chemiluminescent image analysis system (Tanon, Shanghai, China). The areas analyzed by Image J software was used to protein quantification.

Functional binding sites predicted using the JASPAR database

The promoter of *CYP51* was predicted using the online BDGP database (http://www.fruitfly.org/seq_tools/promoter.html). Then, sequence A (a 93 bp nucleotide sequence of *CYP51* promoter) and sequence B (a 95 bp nucleotide sequence of *CYP51* promoter with the 'CC' insertion mutation) were used to predict functional binding sites in the JASPAR database (http://jaspar.cgb.ki.se) (Table 6). The relative profile score threshold was set to 80%.

Supplementary information

Supplementary information accompanies this paper at https://doi.org/10.1186/s42483-020-00062-x.

Additional file 1: Table S1. Prediction of functional binding sites without 'CC' insertion sequences in the predicted promoter region of *CYP51* in *U. virens* using the JASPAR database. **Table S2.** Prediction of functional binding sites with 'CC' insertion sequences in the predicted promoter region of *CYP51* in *U. virens* using the JASPAR database. **Table S3.** Primers used in this study.

Abbreviations

DMIs: Sterol demethylation inhibitors; EC_{50}: The concentration of fungicides resulting in 50% inhibition of mycelia growth; EC_{90}: The concentration of fungicides resulting in 90% inhibition of mycelia growth; PSA: Potato sucrose agar; RT-qPCR: Reverse transcription-quantitative PCR; RF: Resistance factors; RFS: Rice false smut

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Authors' contributions

XP and HC conducted the experiments. All authors analyzed the data. XP, HC and YL wrote and revised the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

Not applicable.

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Consent for publication

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Competing interests

The authors declare that they have no competing interests.

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