Characterization of Sterol Demethylation Inhibitor-Resistant Isolates of *Fusarium asiaticum* and *F. graminearum* Collected from Wheat in China

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ABSTRACT

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Fusarium asiaticum and *F. graminearum* are the primary causal agents of Fusarium head blight (FHB) of wheat in China. In this study, sensitivities of 159 *F. asiaticum* and *F. graminearum* isolates to a benzimidazole fungicide carbendazim (MBC) and to sterol demethylation inhibitors (DMIs) tebuconazole and prochloraz were determined. Among the 159 isolates, 9 were resistant to MBC and designated as MBC-R isolates. Three showed resistance to tebuconazole and prochloraz and designated as DMI-R isolates. There was no cross-resistance between MBC and DMI. Genetic analysis by microsatellite-primed polymerase chain reaction (PCR) showed that MBC-R or DMI-R isolates had different genotypes, which indicated that they originated from different wild-type

parents. Analysis of two 14 α -demethylase (*cyp51*) homologous genes (*cyp51A* and *cyp51B*) showed that the *F. asiaticum* isolates could be distinguished from *F. graminearum* isolates based on the sequence of *cyp51A*. Analysis of deduced amino acid sequence of *cyp51A* and *cyp51B* suggested that no mutations were associated with DMI resistance. Real-time PCR analysis showed that the DMI resistance was not related to the expression of *cyp51A* and *cyp51B* in *F. asiaticum* and *F. graminearum*, but expressions of both genes were induced greatly by the tebuconazole. Results of this study indicated that *cyp51A* would be an informative marker for analysis of population structure of *F. asiaticum* and *F. graminearum*, and the existence of homologous *cyp51* genes in *F. asiaticum* and *F. graminearum* could provide new insights into DMI resistance in phytopathogenic fungi.

Additional keyword: azole.

Fusarium head blight (FHB) is a devastating disease of cereal crops which can cause huge losses in epidemic years. In addition to the yield loss caused by the disease, the contamination of infected grains with mycotoxins poses a serious threat to human and animal health. Although a number of *Fusarium* spp. can cause FHB, the primary etiological agents of this disease belong to the *Fusarium graminearum* species complex of B-trichothecene toxin producers, which contain at least 11 phylogenetic species, including *F. acaciae-mearnsii*, *F. asiaticum*, *F. austro-americanum*, *F. boothii*, *F. brasilicum*, *F. cortaderiae*, *F. gerlachii*, *F. graminearum*, *F. meridionale*, *F. mesoamericanum*, and *F. vorosii*, (11,32,34). Different *Fusarium* spp. may be associated with FHB in different regions of the world because of different cropping systems and climatic conditions (28,36). In China, *F. asiaticum* and *F. graminearum* are the major causal agents of FHB, although other *Fusarium* spp. (such as *F. culmorum*, *F. avenaceum*, and *F. tricinctum*) were also isolated occasionally from infected wheat heads (7). *F. asiaticum* isolates are able to produce deoxynivalenol, 3-acetyldeoxynivalenol (3-AcDON), 15-acetyldeoxynivalenol (15-AcDON), nivalenol, and 4-acetylnivalenol (NIV) mycotoxins. However, *F. graminearum* isolates produce only 15-AcDON and do not produce 3-AcDON or NIV (37). The difference between *F. asiaticum* and *F. graminearum* that produce 15-AcDON is the presence or absence of 11-bp repeats within the sequence of a trichothecene biosynthesis gene *Tri7*. The 11-bp repeats are present in *F. graminearum* but absent in *F. asiaticum* (37). 15-AcDON chemotype carrying the 11-bp repeats belongs to *F. graminearum*, whereas the same chemotype

lacking the 11-bp repeats is of *F. asiaticum* (14,29,37). Zhang et al. (37) analyzed 299 isolates collected from various epidemic regions of China and found that 231 isolates (77.3%) belonged to *F. asiaticum* and the remaining 68 isolates were *F. graminearum*. These two species are also predominant in the populations of FHB pathogens in Japan (33).

Currently, most wheat cultivars planted in the world are susceptible to *F. graminearum* complex; therefore, one primary method for management of FHB is through the application of fungicides during wheat anthesis, the time of primary infection. In China, the benzimidazole fungicides, particularly carbendazim (MBC), have been used extensively for the control of FHB since the early 1970s. Because of the heavy application of benzimidazole fungicides, resistance of *F. graminearum* complex to MBC has been detected since 1992, and a decline in the control of FHB with MBC has been observed in some areas since 1998 (5,35,38). Alternative applications of MBC with other fungicides, therefore, would be necessary in management of MBC resistance in FHB.

The sterol demethylation inhibitor (DMI) fungicides inhibit the sterol C-14 α -demethylation of 24-methylenedihydrolanosterol, a precursor of ergosterol in fungi (4). DMIs comprise a large number of commercially successful fungicides and were first used agriculturally in the 1970s. Many field studies showed that DMI fungicides, such as tebuconazole and prochloraz, are practically effective against *Fusarium* spp. (25,26). Although declining sensitivity to DMI in FHB pathogens has not caused a problem in the field yet (13), DMI resistance has developed in the populations of several important phytopathogenic fungi, including *Blumeriella jaapii* (22), *Erysiphe graminis* (9), *Monilinia fructicola* (18), and *Mycosphaerella graminicola* (15), which indicated that it is necessary to incorporate effective antiresistance strategies in management of FHB with DMIs.

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Molecular mechanisms of DMI resistance in fungi have been investigated intensively. Common mechanisms of DMI resistance include (i) mutations in the DMI target enzyme, 14 α -demethylase (CYP51), encoded by the *cyp51* gene; (ii) overexpression of the *cyp51* gene; and (iii) overexpression of ATP-binding cassette (ABC) transporter genes encoding efflux pumps (10,16,20,30,31). To date, resistance of *F. asiaticum* or *F. graminearum* to DMI fungicides has not been documented. Thus, the major objectives of this study were to (i) determine sensitivity to DMI fungicides tebuconazole and prochloraz in *F. asiaticum* and *F. graminearum* collected from wheat in China and (ii) investigate possible mechanisms of DMI resistance in *F. asiaticum* and *F. graminearum*.

MATERIALS AND METHODS

Determination of *F. asiaticum* and *F. graminearum* sensitivity to MBC, tebuconazole, and prochloraz. In total, 159 single-spore isolates of *F. asiaticum* and *F. graminearum* (118 *F. asiaticum* and 41 *F. graminearum* isolates) used in this study were collected from infected wheat heads in 2007 and 2008 at different locations, where tebuconazole and prochloraz have never been used but MBC and triadimefon have been used for more than 20 years (Table 1). In these locations, triadimefon was used for the control of wheat powdery mildew. The isolates were recovered from individual infected wheat heads and no more than four isolates were collected from each sampled field. *F. asiaticum* and *F. graminearum* were identified based on the presence or absence of 11-bp repeats within the *Tri7* gene detected with the primer pair GzTri7f1/r1 (Table 2) using a previously published protocol (14,37).

MBC (25% active ingredient [a.i.]) (GuoGuang Agricultural Chemical Co. Ltd., Sichuan, China) was dissolved in sterilized water, adjusted to a concentration of 100 mg/ml, and added to potato dextrose agar (PDA) medium after sterilization to produce final concentrations of 0, 10, 50, 100, and 500 μ g/ml. The concentrations were chosen according to a previous study (5). Technical tebuconazole (97.9% a.i.) (Shanghai Agricultural Chemical Co. Ltd., Shanghai, China) was dissolved in acetone at a concentration of 100 mg/ml and added to PDA medium after sterilization to produce final concentrations of 0, 0.175, 0.35, 0.7, 1.4, 2.8, 5.6, and 11.2 μ g/ml. Prochloraz (95% a.i.) (QingFeng Agricultural Chemical Co. Ltd., Zhejiang, China) was dissolved in acetone, adjusted to a concentration of 100 mg/ml, and added to PDA to produce final concentrations of 0, 0.01, 0.03, 0.1, 0.3, 1.0, 3.0, and 9.0 μ g/ml.

To determine sensitivity of *F. asiaticum* and *F. graminearum* to MBC, a 5-mm mycelial plug was taken from the edge of a 3-day-old colony and placed on the center of a PDA plate amended with MBC at each of the above concentrations. Three replicate plates for each concentration were used for each isolate. After the plates were incubated at 25°C for 5 days in the dark, the mycelial growth was recorded for each plate. The experiment was repeated three times.

To determine sensitivity of *F. asiaticum* and *F. graminearum* to tebuconazole and prochloraz, a 5-mm mycelial plug was taken from the edge of a 3-day-old colony and placed on the center of a PDA plate amended with tebuconazole or prochloraz at each of the above concentrations. Two replicates for each concentration were used for each isolate. After incubation at 25°C for 3 days, colony diameter in each plate was measured in two perpendicular directions with the original mycelial plug diameter (5 mm) subtracted from each measurement. For each plate, the average of the colony diameters was used for calculating the fungicide concentration that results in 50% mycelial growth inhibition (EC₅₀). The EC₅₀ values were calculated with the Data Processing System (DPS) computer program (Hangzhou Reifeng Information Technology Ltd., Hangzhou, China). The experiment was per-

formed twice. Because the two experiments did not differ significantly ($P > 0.05$, Fisher's least significant difference test), the average EC₅₀ values from the two experiments for each isolate were used in the data analysis.

Genetic relationships among the MBC-resistant and DMI-resistant isolates. In order to investigate genetic relationships among the MBC-resistant (MBC-R) or DMI-resistant (DMI-R) isolates, all 9 MBC-R, 3 DMI-R, and 27 sensitive isolates were analyzed by microsatellite-primed polymerase chain reaction (MP-PCR) with three microsatellite primers: (AAC)₅, (GACA)₄, and M13 (Table 2). These three primers were chosen because they were informative in previous studies for *Botryosphaeria dothidea* (19) and *Botrytis cinerea* (21). PCR amplifications were performed in a 25- μ l volume containing 20 ng of fungal genomic DNA, 0.2 μ M each primer, 0.2 mM each of dNTP, 2.0 mM MgCl₂, 1 $\times$ Promega Taq polymerase buffer (10 mM Tris-HCl, pH 9.0; 50 mM KCl; and 0.1% Triton X-100), and 2 units of Taq polymerase (Shanghai Promega Corp., Shanghai, China). The PCR amplifications were performed in a PTC-200 DNA Engine Cycler (MJ Research, Waltham, MA, USA) set to the following parameters: an initial preheat for 3 min at 95°C; followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 45°C for 1 min, and extension at 72°C for 1.5 min; and terminated with a final extension at 72°C for 10 min. Each isolate was amplified twice. The PCR products were separated on 1.5% agarose gels in 1 $\times$ Tris-acetate (TAE) buffer and photographed after staining with ethidium bromide.

To analyze MP-PCR data, each isolate was scored for the presence or absence of each amplicon using Gel-Pro System (Media Cybernetics Inc., Bethesda, MD). Genetic similarities (S) were calculated using a simple matching coefficient. A phenogram was constructed using the unweighted pair group method with arithmetic average (UPGMA) by the program Sequential, Agglomerative, Hierarchical, and Nested clustering methods (SAHN) of the software package NTSYS-pc 2.1 (Department of Ecology and Evolution, State University of New York).

Analysis of DNA sequence of *cyp51* genes from DMI-sensitive and DMI-R isolates. To investigate possible mechanisms of *F. asiaticum* and *F. graminearum* resistance to DMIs, DNA sequences of *cyp51* genes from three DMI-R (JS3, JS6, and HN8) and two DMI-sensitive (DMI-S) isolates (HB3 and NX3) were analyzed. The search for *cyp51* in the genome database of *F. graminearum* showed that there were three putative *cyp51* genes, designated as *cyp51A*, *cyp51B*, and *cyp51C*. Based on the sequences deposited on the genome database of *F. graminearum* at Broad Institute (available online), the primer pairs F51A-F + F51A-R, F51B-F + F51B-R, and F51C-F1 + F51C-R1 (Table 2) were designed to amplify the full sequence of *cyp51A*, *cyp51B*, and *cyp51C*, respectively. PCR amplifications were performed using the following parameters: initial preheat for 3 min at 95°C; followed by 35 cycles of 94°C for 1 min, 53°C for 30 s, and 72°C for 1 min; and a final extension at 72°C for 5 min. The PCR product from each isolate was purified, cloned, and sequenced. The experiment was conducted independently twice for each isolate to avoid mismatch sequences during PCR amplification and sequencing. The sequences were deposited into GenBank under accession numbers FJ216393 to FJ216402.

Analysis of expression of *cyp51* genes in DMI-R and DMI-S isolates. To determine possible differences in expression of *cyp51* genes between DMI-R and DMI-S isolates, each of DMI-R isolates JS3, JS6, and HN8 and DMI-S isolates HB3 and HE-3 was cultured in potato dextrose broth (PDB) medium for 4 days. Before mycelia were harvested for RNA extraction, each isolate was treated with tebuconazole at 2.5 μ g/ml for 6 h. The untreated mycelia of each isolate were used as the control. RNA from mycelia of each isolate was extracted using a TaKaRa RNAiso Reagent (TaKaRa Biotech. Co., Dalian, China) according to the manufacturer's instructions. Briefly, fresh mycelia of each sample

TABLE 1. Sensitivity of *Fusarium asiaticum* and *F. graminearum* isolates to a benzimidazole fungicide, carbendazim, and demethylation inhibitors tebuconazole and prochloraz

Isolate	Year of isolation	Origin (city, province)	EC ₅₀ (µg/ml) ^a		Sensitivity to carbendazim ^b
			Tebuconazole	Prochloraz	
<i>F. asiaticum</i>					
JS1	2007	Zhenjiang, Jiangsu	0.770	0.040	MBC-S
JS2	2007	Zhenjiang, Jiangsu	0.297	0.060	MBC-S
JS3	2007	Zhenjiang, Jiangsu	4.152	1.467	MBC-S
JS4	2007	Zhenjiang, Jiangsu	0.109	0.020	MBC-S
JS5	2007	Yangzhou, Jiangsu	0.126	0.013	MBC-S
JS6	2007	Yangzhou, Jiangsu	4.517	2.888	MBC-S
JS7	2007	Yangzhou, Jiangsu	0.124	0.035	MBC-S
JS8	2007	Yangzhou, Jiangsu	0.097	0.036	MBC-S
JS9	2007	Yangzhou, Jiangsu	0.085	0.029	MBC-S
JS10	2008	Huai'an, Jiangsu	0.064	0.071	MBC-S
JS11	2008	Huai'an, Jiangsu	1.236	0.154	MBC-S
JS12	2008	Huai'an, Jiangsu	0.460	0.032	MBC-S
JS13	2008	Huai'an Jiangsu	0.938	0.021	MBC-S
JS14	2007	Lianyungang, Jiangsu	0.254	0.027	MBC-S
JS15	2007	Lianyungang, Jiangsu	0.064	0.036	MBC-S
JS16	2007	Lianyungang, Jiangsu	0.085	0.036	MBC-S
JS17	2007	Taizhou, Jiangsu	0.075	0.020	MBC-R
JS18	2007	Taizhou, Jiangsu	0.062	0.029	MBC-R
JS19	2007	Taizhou, Jiangsu	0.095	0.065	MBC-S
JS20	2007	Taizhou, Jiangsu	0.252	0.018	MBC-S
JS21	2007	Zhangjiagang, Jiangsu	0.202	0.030	MBC-S
JS22	2007	Nanjing, Jiangsu	0.692	0.047	MBC-S
JS23	2007	Nantong, Jiangsu	0.138	0.036	MBC-S
JS24	2007	Nantong, Jiangsu	0.089	0.038	MBC-R
JS25	2007	Nantong, Jiangsu	0.102	0.065	MBC-R
JS26	2007	Nantong, Jiangsu	0.055	0.031	MBC-R
JS27	2007	Nantong, Jiangsu	1.236	0.154	MBC-R
AH1	2008	Chuzhou, Anhui	2.111	0.570	MBC-S
AH3	2008	Chuzhou, Anhui	0.161	0.014	MBC-S
AH4	2008	Chuzhou, Anhui	0.161	0.007	MBC-S
AH5	2008	Chuzhou, Anhui	0.065	0.015	MBC-S
AH6	2008	Chuzhou, Anhui	0.179	0.017	MBC-S
AH7	2008	Chuzhou, Anhui	0.096	0.024	MBC-S
AH8	2008	Chuzhou, Anhui	0.055	0.015	MBC-S
AH9	2008	Chuzhou, Anhui	0.204	0.023	MBC-S
AH10	2008	Hefei, Anhui	0.306	0.025	MBC-S
AH11	2008	Hefei, Anhui	0.834	0.024	MBC-S
AH12	2008	Hefei, Anhui	0.527	0.065	MBC-S
AH13	2008	Liu'an, Anhui	0.032	0.012	MBC-S
AH14	2008	Liu'an, Anhui	0.016	0.013	MBC-S
AH15	2008	Liu'an, Anhui	0.549	0.089	MBC-S
AH16	2008	Fuyang, Anhui	0.984	0.090	MBC-S
AH17	2008	Suzhou, Anhui	0.554	0.015	MBC-S
AH19	2008	Ningguo, Anhui	0.043	0.002	MBC-S
AH20	2008	Ningguo, Anhui	1.444	0.156	MBC-S
AH21	2008	Huainan, Anhui	1.345	0.113	MBC-S
AH22	2008	Huainan, Anhui	0.168	0.023	MBC-S
AH23	2008	Wuhu, Anhui	0.134	0.033	MBC-S
AH24	2008	Wuhu, Anhui	0.245	0.052	MBC-S
HB1	2007	Xiaogan, Hubei	1.505	0.206	MBC-S
HB2	2007	Xiaogan, Hubei	0.213	0.016	MBC-S
HB3	2007	Xiaogan, Hubei	0.031	0.019	MBC-S
HB4	2007	Xiaogan, Hubei	0.199	0.167	MBC-S
HB5	2007	Xiaogan , Hubei	0.122	0.112	MBC-S
HB6	2007	Xiaogan , Hubei	0.333	0.111	MBC-S
HB7	2007	Xiaogan , Hubei	0.174	0.022	MBC-S
HB8	2007	Xiaogan , Hubei	0.101	0.040	MBC-S
HB9	2007	Xiaogan, Hubei	0.190	0.016	MBC-S
HB10	2007	Shiyan, Hubei	0.143	0.017	MBC-S
HB12	2007	Shiyan, Hubei	0.063	0.237	MBC-S
HB13	2007	Shiyan, Hubei	0.533	0.015	MBC-S
HB14	2007	Zhongxiang, Hubei	0.088	0.020	MBC-S
HB15	2007	Xiangfan, Hubei	0.116	0.049	MBC-S
HB16	2007	Xiangfan, Hubei	0.317	0.067	MBC-S
HB17	2007	Jingmen, Hubei	0.147	0.025	MBC-S
HB18	2007	jingmen, Hubei	0.813	0.020	MBC-S
HB19	2007	Qianjiang, Hubei	0.163	0.018	MBC-S
HB20	2007	Qianjiang, Hubei	0.174	0.026	MBC-S

(continued on next page)

^a EC₅₀ = effective concentration that results in 50% mycelial growth inhibition.

^b MBC-S and MBC-R indicate carbendazim (MBC)-sensitive and -resistant isolates, respectively.

TABLE 1. (continued from preceding page)

Isolate	Year of isolation	Origin (city, province)	EC ₅₀ (µg/ml) ^a		Sensitivity to carbendazim ^b
			Tebuconazole	Prochloraz	
HB21	2007	Wuchang, Hubei	0.190	0.026	MBC-S
HB22	2007	Wuchang, Hubei	0.288	0.035	MBC-S
HB23	2007	Wuchang, Hubei	0.092	0.043	MBC-S
HB24	2007	Wuhan, Hubei	1.717	0.302	MBC-S
JX1	2007	Jiujiang, Jiangxi	0.248	0.056	MBC-S
JX2	2007	Jiujiang, Jiangxi	0.085	0.115	MBC-S
JX3	2007	Jiujiang, Jiangxi	2.079	0.376	MBC-S
JX4	2007	Jiujiang, Jiangxi	0.090	0.035	MBC-S
JX5	2007	Jiujiang, Jiangxi	0.086	0.055	MBC-S
JX6	2007	Jiangxi	0.130	0.094	MBC-S
JX7	2007	De'an, Jiangxi	0.093	0.050	MBC-S
JX8	2007	De'an, Jiangxi	0.111	0.050	MBC-S
JX10	2007	Ruichang, Jiangxi	0.249	0.047	MBC-S
ZJ1	2008	Wenzhou, Zhejiang	0.213	0.015	MBC-S
ZJ2	2008	Wenzhou, Zhejiang	0.097	0.007	MBC-S
ZJ3	2008	Wenzhou, Zhejiang	0.395	0.024	MBC-S
ZJ4	2008	Wenzhou, Zhejiang	0.087	0.003	MBC-S
ZJ5	2008	Wenzhou, Zhejiang	0.097	0.001	MBC-S
ZJ6	2008	Fuyang, Zhejiang	0.130	0.036	MBC-S
ZJ7	2008	Fuyang, Zhejiang	0.221	0.023	MBC-S
ZJ8	2008	Hangzhou, Zhejiang	0.328	0.032	MBC-S
ZJ9	2008	Hangzhou, Zhejiang	0.206	0.056	MBC-S
ZJ10	2008	Hangzhou, Zhejiang	0.089	0.002	MBC-R
ZJ11	2008	Pinghu, Zhejiang	0.896	0.285	MBC-R
ZJ12	2008	Pinghu, Zhejiang	1.230	0.153	MBC-R
ZJ13	2008	Pinghu, Zhejiang	0.097	0.005	MBC-S
ZJ14	2008	Pinghu, Zhejiang	0.174	0.024	MBC-S
GZ1	2007	Guiyang, Guizhou	0.174	0.008	MBC-S
GZ2	2007	Tongren, Guizhou	0.323	0.018	MBC-S
GZ3	2007	Tongren, Guizhou	0.164	0.024	MBC-S
GZ4	2007	Tongren, Guizhou	0.167	0.015	MBC-S
FJ1	2007	Jianyang, Fujian	0.145	0.002	MBC-S
SC1	2007	Deyang, Sichuan	0.670	0.022	MBC-S
SC2	2007	Deyang, Sichuan	0.108	0.032	MBC-S
SC4	2007	Ya'an, Sichuan	0.096	0.067	MBC-S
HE-5	2007	Handan, Hebei	0.211	0.423	MBC-S
HN6	2007	Luohe, Henan	0.150	0.024	MBC-S
HN11	2007	Luohe, Henan	0.042	0.003	MBC-S
HN13	2007	Zhengzhou, Henan	0.089	0.002	MBC-S
HN23	2007	Jiaozuo, Henan	0.089	0.009	MBC-S
SD2	2007	Zaozhuang, Shandong	0.111	0.022	MBC-S
SD3	2007	Zaozhuang, Shandong	0.347	0.280	MBC-S
SD5	2007	Zaozhuang, Shandong	0.494	0.031	MBC-S
HLJ3	2007	Daqing, Heilongjiang	0.187	0.002	MBC-S
HLJ6	2007	Daqing, Heilongjiang	0.010	0.018	MBC-S
HLJ7	2007	Daqing, Heilongjiang	0.065	0.002	MBC-S
HLJ8	2007	Daqing, Heilongjiang	0.137	0.014	MBC-S
NX4	2007	Yinchuan, Ningxia	0.073	0.015	MBC-S
SX1	2007	Shangluo, Shanxi	0.110	0.020	MBC-S
SX2	2007	Shangluo, Shanxi	0.667	0.246	MBC-S
<i>F. graminearum</i>					
AH2	2008	Chuzhou, Anhui	0.021	0.009	MBC-S
AH18	2008	Suzhou, Anhui	0.562	0.073	MBC-S
HB11	2007	Shiyan, Hubei	0.700	0.025	MBC-S
JX9	2007	Ruichang, Jiangxi	0.082	0.033	MBC-S
SC3	2007	Deyang, Sichuan	0.443	0.013	MBC-S
HE-1	2007	Handan, Hebei	0.046	0.002	MBC-S
HE-2	2007	Handan, Hebei	0.516	0.021	MBC-S
HE-3	2007	Handan, Hebei	0.059	0.010	MBC-S
HE-4	2007	Handan, Hebei	0.050	0.014	MBC-S
HN1	2008	Luohe, Henan	0.069	0.027	MBC-S
HN2	2008	Luohe, Henan	1.098	0.032	MBC-S
HN3	2008	Luohe, Henan	0.120	0.012	MBC-S
HN4	2008	Luohe, Henan	0.436	0.077	MBC-S
HN5	2008	Luohe, Henan	0.164	0.030	MBC-S
HN7	2008	Luohe, Henan	0.123	0.030	MBC-S
HN8	2008	Luohe, Henan	6.235	4.575	MBC-S
HN9	2008	Luohe, Henan	0.144	0.010	MBC-S
HN10	2008	Luohe, Henan	0.034	0.001	MBC-S
HN12	2008	Luohe, Henan	0.156	0.089	MBC-S
HN14	2008	Luoyang, Henan	0.103	0.014	MBC-S

(continued on next page)

(approximately 100 mg) were ground with a motor-driven pestle in 100 µl of TaKaRa RNAiso Reagent in a 1.5-ml centrifuge tube for 1 min; then, 900 µl of RNAiso Reagent was added. After the tubes were kept at 22 ± 2°C (room temperature) for 5 min, 200 µl of chloroform was added into each tube to emulsify. After incubation at room temperature for 5 min, the tubes were spun for 15 min at 12,000 rpm. The supernatants were transferred into new tubes and an equal volume of isopropanol was added to precipitate the RNA. The tubes were kept at room temperature for 10 min before being spun at 10,000 rpm for 15 min. Finally, recovered RNA pellets were washed with 75% ethanol and suspended in 20 µl of RNase-free water.

For each RNA sample, 4 µl of RNA solution was used for reverse transcription using the oligo(dT)₁₈ primer with a RevertAid H Minus First Strand cDNA Synthesis kit (Fermentas Life Sciences, Burlington, Canada) according to the manufacturer's recommendations. Reverse-transcription PCRs with the primer pairs F51A-EX-F + F51A-EX-R and F51B-EX-F + F51B-EX-R (Table 2) were conducted to examine transcriptions of *cyp51A* and *cyp51B*, respectively. Each primer pair spans one intron to identify amplification of possible contaminating genomic DNA. PCR amplifications were performed using the following parameters: initial preheat for 3 min at 95°C; followed by 35 cycles of 94°C for 30 s, 53°C for 30 s, and 72°C for 30 s; and a final extension at 72°C for 5 min.

TABLE 1. (continued from preceding page)

Isolate	Year of isolation	Origin (city, province)	EC ₅₀ (µg/ml) ^a		Sensitivity to carbendazim ^b
			Tebuconazole	Prochloraz	
HN15	2008	Luoyang, Henan	0.036	0.024	MBC-S
HN16	2008	Jiaozuo, Henan	0.242	0.016	MBC-S
HN17	2008	Jiaozuo, Henan	0.356	0.019	MBC-S
HN18	2008	Jiaozuo, Henan	0.481	0.023	MBC-S
HN19	2008	Jiaozuo, Henan	0.231	0.036	MBC-S
HN20	2008	Jiaozuo, Henan	0.456	0.012	MBC-S
HN21	2008	Jiaozuo, Henan	0.295	0.029	MBC-S
HN22	2008	Jiaozuo, Henan	0.045	0.008	MBC-S
SD1	2007	Zaozhuang, Shandong	0.463	0.009	MBC-S
SD4	2007	Zaozhuang, Shandong	0.053	0.002	MBC-S
HLJ1	2007	Ha'erbing, Heilongjiang	0.097	0.011	MBC-S
HLJ2	2007	Ha'erbing, Heilongjiang	0.223	0.021	MBC-S
HLJ4	2007	Daqing, Heilongjiang	0.097	0.001	MBC-S
HLJ5	2007	Daqing, Heilongjiang	0.097	0.006	MBC-S
HLJ9	2007	Daqing, Heilongjiang	0.082	0.033	MBC-S
HLJ10	2007	Daqing, Heilongjiang	1.152	0.071	MBC-S
HLJ11	2007	Daqing, Heilongjiang	0.171	0.010	MBC-S
NX1	2007	Yinchuan, Ningxia	0.068	0.010	MBC-S
NX2	2007	Yinchuan, Ningxia	0.121	0.036	MBC-S
NX3	2007	Yinchuan, Ningxia	0.046	0.010	MBC-S
SX3	2007	Shangluo, Shanxi	0.181	0.032	MBC-S

TABLE 2. List of polymerase chain reaction (PCR) primers used in this study

Primer	Sequence (5'–3')	Relevant characteristics ^a
GzTri7f1	GCTTTACGACTCCTCAACAATGG	PCR primer pairs used for the identification of <i>F. asiaticum</i> and <i>F. graminearum</i> isolates
GzTri7r1	AGAGCCCTGCGAAAG(C/T)ACTGGTGC	
(AAC) ₅	AACAACAACAACAAC	Microsatellite primers for analysis of genetic variation in population of <i>F. asiaticum</i> and <i>F. graminearum</i>
(GACA) ₄	GACAGACAGACAGACA	
M13	GAGGGTGGCGGTTCT	PCR primers for amplification of full sequence of <i>cyp51A</i> gene from DMI-R and DMI-S isolates of <i>F. asiaticum</i> and <i>F. graminearum</i>
F51A-F	TTCCAATCTCTGTGTTTTCGCT	
F51A-R	TCGGGATGCTAACACAACCTT	PCR primers for amplification of full sequence of <i>cyp51B</i> gene from DMI-R and DMI-S isolates of <i>F. asiaticum</i> and <i>F. graminearum</i>
F51B-F	GGTCCGAAAAAAGATGTTCCG	
F51B-R	ATCATTGCCGTCAGGCATAA	PCR primer pairs for amplification of <i>cyp51C</i> gene from DMI-R and DMI-S isolates of <i>F. asiaticum</i> and <i>F. graminearum</i>
F51C-F1	AGCACGATGGAATCGCTCTA	
F51C-R1	GCTCGGTCTACACATCCATCA	PCR primers for analysis expression of <i>cyp51A</i> gene in DMI-R and DMI-S isolates of <i>F. asiaticum</i> and <i>F. graminearum</i>
F51C-F2	ATGGAATCGCTCTACGAGACT	
F51C-R2	TCATTCTACTGTCTCGCGTCG	
F51C-F3	TTACGTTCAACGACCTGGA	
F51C-R3	AGACGAAAGCAGTGTGTGGGA	
F51C-F4	TCACAATCTACACAGCATCCG	
F51C-R4	TCCTCGTACAGTTCCTGAACA	
F51C-F5	TGGAATCGCTCTACGAGACTC	
F51C-R5	AGCATGGTTTCGACGGAAGT	
F51A-EX-F	TGGACCCTTGTGGCTTCTTT	
F51A-EX-R	GCTTTTTGCGTAAGGCCAAA	
F51B-EX-F	TCAATGTCTCAACCAGCTC	
F51B-EX-R	AGGATGAAGTCGTTTCCAGCA	
F-actin-F	ATCCACGTCACCACTTTCAA	
F-actin-R	TGCTTGAGATCCACATTTG	

^a DMI-R and DMI-S = demethylation inhibitor-resistant and -sensitive, respectively.

Expression levels of *cyp51A* and *cyp51B* in each DMI-R or DMI-S isolate were determined by real-time PCR assays. The real-time PCR amplifications were performed in a DNA Engine Opticon 4 System (MJ Research) using the SYBR Green I fluorescent dye detection. Amplifications were conducted in a 20- μ l volume containing 10 μ l of iQ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA), 1 μ l of reverse transcription product, and 0.5 μ l each of the forward and reverse primers (10 pmol each). There were two replicates for each sample. The real-time PCR amplifications were performed with the following parameters: an initial preheat at 95°C for 2 min, followed by 35 cycles at 95°C for 15 s, 53°C for 20 s, 72°C for 40 s, and 75°C for 3 s in order to detect and quantify the fluorescence at a temperature above the denaturation of primer-dimers. Once amplifications were completed, melting curves were obtained based on a standard protocol and used to identify PCR products. For each sample, PCR amplifications with primer pair F-actin-F + F-actin-R (Table 2) for the quantification of expression of actin gene were performed as a reference because the actin is constitutively expressed (27). The real-time PCR conditions for the primers F-actin-F + F-actin-R were the same as those of the primer pair F51A-EX-F + F51A-EX-R. The experiment was repeated twice. The expression of *cyp51A* and *cyp51B* in the fungicide-treated sample relative to that of untreated sample was calculated using the $2^{-\Delta\Delta C_t}$ method (17) and the data were analyzed using analysis of variance of SAS (version 8.0; SAS Institute, Cary, NC).

RESULTS

Sensitivity of *F. asiaticum* and *F. graminearum* to MBC.

Previous studies showed that MBC at 10 μ g/ml could be used as the threshold concentration for monitoring MBC resistance in *F. graminearum* (35). In this study, among the 118 *F. asiaticum* and 41 *F. graminearum* isolates tested, 109 *F. asiaticum* isolates and all 41 *F. graminearum* isolates were unable to grow on PDA amended with MBC at 10 μ g/ml; therefore, they were designated

Fig. 1. Frequency distribution of effective concentration that results in 50% mycelial growth inhibition (EC₅₀) of **A**, tebuconazole and **B**, prochloraz for 118 *Fusarium asiaticum* isolates and 41 *F. graminearum* isolates collected from different locations of China.

as MBS-sensitive (MBC-S) isolates. Nine *F. asiaticum* isolates were able to grow on PDA amended with MBC at 100 μ g/ml but not at 500 μ g/ml within 5 days of incubation. These isolates were designated as MBC-R isolates (Table 1). All nine MBC-R isolates were collected from Zhejiang and Jiangsu provinces. MBC-R isolates were not detected from the samples collected from any of the other 12 provinces of China.

Sensitivity of *F. asiaticum* and *F. graminearum* to tebuconazole and prochloraz. The EC₅₀ values for tebuconazole for the *F. graminearum* isolate HN8 and *F. asiaticum* isolates JS3 and JS6 were 6.24, 4.152, and 4.52 μ g/ml, respectively, which were significantly ($P < 0.001$, $F = 388.66$) higher than those of the other 156 tested isolates. Thus, these three isolates were designated as DMI-R isolates and the rest were DMI-S. Except for these three isolates, the EC₅₀ values of 40 *F. graminearum* isolates to tebuconazole were 0.021–1.152 μ g/ml, with a mean of 0.248 μ g/ml. The EC₅₀ values of the 116 *F. asiaticum* isolates to tebuconazole were 0.010–2.111 μ g/ml, with a mean of 0.316 μ g/ml (Table 1). Because the *F. graminearum* and *F. asiaticum* isolates were not significantly different ($P = 0.9936$) in their sensitivity to tebuconazole, they were combined for analysis of the frequency distribution of EC₅₀ values to this fungicide (Fig. 1A). Inoculation tests showed that all three DMI-R isolates were able to cause disease on wheat heads treated with tebuconazole at 50 μ g/ml but sensitive isolates HB3 and NX6 did not cause any disease.

Similar to tebuconazole, the three DMI-R isolates HN8, JS3, and JS6 were also resistant to prochloraz, with EC₅₀ values of 4.575, 1.467, and 2.888 μ g/ml, respectively. The EC₅₀ values were significantly ($P < 0.001$, $F = 678.68$) higher than those of the remaining 156 *F. asiaticum* and *F. graminearum* isolates, which indicated that there was a cross-resistance between tebuconazole and prochloraz. For the rest of the 156 *F. asiaticum* and *F. graminearum* isolates, the EC₅₀ values for prochloraz were 0.001 to 0.570 μ g/ml, with a mean of 0.050 μ g/ml. The frequency distribution of EC₅₀ values of the 159 *F. asiaticum* and *F. graminearum* isolates for prochloraz is presented in Figure 1B.

Genetic relationships among the MBC-R and DMI-R isolates. Three microsatellite primers generated a total of 22 polymorphic fragments from 39 *F. asiaticum* and *F. graminearum* isolates. Examples of DNA fingerprint pattern generated by the primer (AAC)₅ are presented in Figure 2. UPGMA analysis showed that the nine MBC-R isolates were clustered into different groups. The three DMI-R isolates were also clustered into different groups (Fig. 3). These results indicated that MBC-R isolates and DMI-R isolates originated from different wild-type parents. Additionally, most *F. asiaticum* and *F. graminearum* isolates were clustered in separated groups (Fig. 3), which indicated that these microsatellite primers could generate informative markers for analysis of *F. graminearum* complex.

Comparison in DNA sequence of *cyp51A* and *cyp51B* genes from DMI-S and DMI-R isolates. Primer pair F51C-F1 + F51C-R1 amplified a target fragment from each *F. graminearum* isolate but not from any *F. asiaticum* isolate tested; therefore, four other primer pairs based on the DNA sequence of *F. graminearum* *cyp51C* were designed (Table 2). However, these four primer pairs did not amplify any fragment from *F. asiaticum*. Thus, in this study, we only analyzed two *cyp51* genes, *cyp51A* and *cyp51B*.

The DNA sequences of *cyp51A* and *cyp51B* from the *F. graminearum* isolate HN8 had 99.94 and 99.89% similarity, respectively, to those of the isolate PH-1, whose genome has been sequenced. The deduced amino acid sequence of *cyp51A* from the isolate HN8 was 64.66 and 59.31% to that of *Aspergillus fumigatus* (GenBank accession no. AF338659.1) and *Penicillium digitatum* (AB030178.1), respectively. The CYP51B from HN8 was also homologous to those from other phytopathogenic fungi. The deduced amino acid of *cyp51B* from HN8 showed similarities of 68.69 and 67.17% to that from *Monilinia fructicola*

(AF470621.1) and *B. cinerea* (AF346594.1), respectively. The percentages of identity of the amino acid sequence were high enough to consider *cyp51A* and *cyp51B* to be members of the fungal *cyp51* family (23) (Fig. 4).

Phylogenetic analysis of DNA sequence of *cyp51A* and *cyp51B* showed that the *F. asiaticum* and *F. graminearum* isolates were clustered in two separate groups (Fig. 5A and B). In all, 69 nucleotide (nt) differences were observed in DNA sequence of the *cyp51A* between these two species. Among these 69 nt differences, 53 did not cause any changes in deduced amino acids (silent substitutions) and the remaining 16 caused 16 amino acid differences that distinguished *F. asiaticum* from *F. graminearum* (Fig. 6, arrows). For DNA sequence of *cyp51B* gene, 4 nt differences were observed between these two species; only 1 nt caused one amino acid change at the codon position 30 whereas the other 3 nt were silent substitutions. These results indicated that *cyp51A* could be an informative marker for the differentiation of phylogenetic species in *F. graminearum* complex.

Comparisons of the deduced amino acid sequence of CYP51A for the different isolates of *F. asiaticum* or *F. graminearum*

showed that the three *F. asiaticum* isolates (JS3, JS6, and HB3) had three different amino acids and the three *F. graminearum* isolates (HN8, NX3, and PH-1) had five differences. However, the differences were not associated with DMI resistance (Fig. 6). Similar to those in the *cyp51A* gene, none of the different amino acids in *cyp51B* was found to be associated with DMI resistance.

Expression of *cyp51* genes in DMI-R and DMI-S isolates. Reverse-transcription PCR with primer pair F51A-EX-F + F51A-EX-R for *cyp51A* and F51B-EX-F + F51B-EX-R for *cyp51B* showed that both genes were transcribed in *F. asiaticum* and *F. graminearum*. Real-time PCR assays showed that the expression levels of both *cyp51A* and *cyp51B* were significantly ($P = 0.0053$ for *cyp51A*; $P = 0.0002$ for *cyp51B*) different among five tested isolates but were not associated with DMI resistance (Fig. 7A and B). When the isolates were treated with tebuconazole at 2.5 µg/ml 6 h before RNA extraction, expressions of *cyp51A* and *cyp51B* were higher than those of the isolates without fungicide treatment. The results indicated that expressions of both genes were induced greatly by tebuconazole.

Fig. 2. Electrophoretic separation of DNA fingerprints of *Fusarium asiaticum* (JS3, JS6, JX3, JX4, HB1, HB3, and HB12) and *F. graminearum* isolates (SD4, HN8, HLJ10, SX3, and NX3) amplified by the microsatellite primer (AAC)₅.

Fig. 3. Unweighted pair group method with arithmetic averages cluster analysis of microsatellite-primed polymerase chain reaction data for 30 *Fusarium asiaticum* and 9 *F. graminearum* isolates, including 9 resistant to carbendazim (MBC-R) and 3 resistant to demethylation inhibitor (DMI-R) fungicides. The remaining 27 isolates were sensitive to both MBC and DMI fungicides.

DISCUSSION

The inexpensive and effective benzimidazole fungicide MBC has been extensively applied for the control of FHB for more than two decades in China. In this study, among 159 isolates, 9 were resistant to MBC. All nine MBC-R isolates were sensitive to DMIs and vice versa; all three DMI-R isolates were sensitive to MBC. The results indicated that alternative applications of benzimidazole and DMI fungicides would be effective in management of MBC resistance in FHB.

In Jiangsu province, MBC-R *F. asiaticum* was first detected in 1994 with a frequency of 0.29%. In 2003, the frequency of resistant isolates increased to 18.69% (38). Since then, most growers used the mixture of MBC, thiram, and triadimefon for control of FHB because the application of MBC alone was not effective. After the mixture was used for a few years, we found that the frequency of resistant isolates was decreased to 7.6% in 2008. In Zhejiang province, the frequency of MBC-R *F. asiaticum* was 0.25% in 1992 but increased to 25.60% in 1999 (38). In the past few years, many farmers did not grow wheat in this area. Subsequently, MBC was not used extensively any longer. In 2008, we tested 139 isolates collected from this area, and found that only three isolates (2.15%) were resistant to MBC (*unpublished data*). These results suggested that the MBC-R *F. asiaticum* isolates might have lower fitness than sensitive ones in nature, and the mixture of MBC with another group of fungicides could delay the development of MBC resistance in *F. asiaticum*.

In this study, the DMI resistance was found from both *F. asiaticum* and *F. graminearum* but MBC resistance was found only from *F. asiaticum*, not from *F. graminearum*. The difference might be explained by the fact that *F. asiaticum* is predominant in

warmer regions (such as Zhejiang and Jiangsu provinces), where FHB occurs frequently, and MBC had been used extensively in these regions. *F. graminearum* is more common than *F. asiaticum* in cooler regions where FHB is not very serious in most years (37). Additionally, although tebuconazole and prochloraz have never been used in the fields where samples were collected, another DMI fungicide, triadimefon, has been used for the control of wheat powdery mildew in most areas of China for more than 20 years. When farmers use triadimefon for the control of wheat powdery mildew, the fungicide may also have resulted in a selection pressure among FHB pathogens. Thus, it would not be surprising that three tebuconazole- and prochloraz-resistant isolates of *F. asiaticum* and *F. graminearum* were detected in this study.

Resistance to benzimidazole fungicides has been found in many fungal species. In most cases, resistance was correlated with point mutations in the β -tubulin gene, which results in altered amino acid sequences at the benzimidazole-binding site. In *F. graminearum* complex, however, the resistant and sensitive isolates had identical deduced amino acid sequence of β -tubulin genes (5). Additionally, Kawchuk et al. (12) reported that the amino acid sequences of the β -tubulin genes from several thiabendazole-resistant and -sensitive isolates of *Gibberella pulicaris* were also identical. Linkage analysis confirmed that the β -tubulin gene was not linked to thiabendazole resistance. These results indicated that other mechanisms than mutation in the β -tubulin should be responsible for the benzimidazole resistance in *Fusarium* spp. More recently, when Chen et al. (6) analyzed a second β -tubulin gene (*tub2*) in *F. graminearum* complex, they found that MBC-R isolates had a point mutation at codon 167, resulting in a Phe replaced by Tyr (F167Y), or a point mutation at the codon position 198, resulting in a Glu replaced by Gln (E198Q). In this study, the point mutations F167Y and E198Q were also detected in five and four MBC-R isolates, respectively.

Our results showed that *F. asiaticum* and *F. graminearum* have homologous *cyp51* genes. The presence of different *cyp51* genes in a phytopathogenic fungus is not common but has been reported from several *Aspergillus* spp. (23). In *A. fumigatus*, the *cyp51A* disrupted mutants were 17- to 40-fold more sensitive than the wild-type parents but they were morphologically indistinguishable from the wild-type parents. The results indicated that *cyp51A* was associated with DMI sensitivity in *A. fumigatus* but it was not essential for viability (24). Interestingly, the disruption of *cyp51A*

Fig. 4. Phylogenetic tree generated by the unweighted pair group method with arithmetic averages on the basis of deduced amino acid sequences of *cyp51A* (FgCYP51A) and *cyp51B* (FgCYP51B) from *Fusarium graminearum* isolate HN8, *cyp51A* (FaCYP51A) and *cyp51B* (FaCYP51B) from *F. asiaticum* isolate JS6, and those from *Aspergillus fumigatus* (AfCYP51A, GenBank accession no. AF338659.1), *Penicillium digitatum* (PdCYP51, AB030178.1), *P. italicum* (PiCYP51, Z49750.1), *A. fumigatus* (AfCYP51B, AF338660.1), *Botrytis cinerea* (BcCYP51, AF346594.1), *Monilinia fructicola* (MfCYP51, AF470621.1), *Tapesia acuformis* (TaCYP51, AF208657.1), *T. yallundae* (TyCYP51, AF208658.2), *Blumeria graminis* (BgCYP51, AF052515.1), *Uncinula necator* (UnCYP51, U83840.2), *Mycosphaerella graminicola* (MgCYP51, AF263470.1), and *Candida albicans* (CaCYP51, AB006854.1).

Fig. 5. Phylogenetic tree generated by the neighbor-joining method on the basis of DNA sequences of **A**, *cyp51A* and **B**, *cyp51B* from the *Fusarium asiaticum* (*Fa*) isolates JS3, JS6, and HB3 and *F. graminearum* (*Fg*) isolates HN8, NX3, and PH-1. Bar indicates 0.05 substitutions per nucleotide position.

did not change the expression level of *cyp51B*. Disruption of *cyp51B* showed that this gene was related to growth rate and shape maintenance but its role in azole susceptibility remains unclear (2). In this study, we found that both *F. asiaticum* and *F. graminearum* have homologous *cyp51* genes but the functions of these homologous *cyp51* genes are not clear. It would be interesting to construct *cyp51A*, *cyp51B*, or *cyp51C* disruption mutants for functional analysis of these genes. *Saccharomyces cerevisiae*

has only one *cyp51* gene, and disruption of *cyp51* gene in this fungus has been described as lethal (3). *F. asiaticum* and *F. graminearum* have three homologous *cyp51* genes; to analyze the function of each gene, we constructed disruptants for each *cyp51* gene. Our preliminary results indicated that none of the proteins are individually essential for *F. asiaticum* and *F. graminearum* viability; however, the *cyp51A* and *cyp51B* disruption mutants showed different sensitivity patterns to different DMI fungicides.

Fig. 6. Alignment of deduced amino acid of *cyp51A* from *Fusarium asiaticum* isolates JS3, JS6, and HB3 and *F. graminearum* isolates HN8, NX3, and PH-1. The JS3, JS6, and HN8 were demethylation inhibitor-resistant (DMI-R) isolates, and the HB3, NX6, and PH-1 were DMI-sensitive (DMI-S). Identical residues are marked by short dash. Different residues between *F. asiaticum* and *F. graminearum* are indicated by short arrows.

The *cyp51A* disruption mutants increased sensitivity significantly to triadimefon and propiconazole but not to tebuconazole and prochloraz (X. Liu and Z. Ma, unpublished data). Thus, it is possible that the different DMI fungicides may target different CYP51 proteins in *F. asiaticum* and *F. graminearum*. These results are in agreement with a previous finding that *F. graminearum* isolates do not show cross-resistance to some of the DMI fungicides (8,13).

In this study, we observed that tebuconazole at 5.6 µg/ml could completely inhibit mycelial growth of DMI-S isolates within 5 days of incubation. However, all tested DMI-S isolates could grow at this concentration after 10 days of incubation. This phenomenon is consistent with the finding that expressions of both *cyp51A* and *-B* genes were induced greatly by the tebuconazole. The DMIs are fungistatic but do not kill cells. When tebuconazole induces *cyp51* expression for a period of time, more target CYP51 proteins are translated (30). Therefore, mycelial growth can occur after a few days of incubation on PDA amended with tebuconazole.

Point mutation in *cyp51* and overexpression of the gene are the common mechanisms of DMI resistance in fungi. In this study, when the DNA sequences of *cyp51A* and *cyp51B* from DMI-S isolates and those from DMI-R isolates were compared, we did not observe any changes associated with DMI resistance. Real-time PCR assays showed that DMI resistance also was not related with levels of *cyp51A* or *cyp51B* expression. Fungicide sensitivity tests showed that although DMI-R isolates were sensitive to MBC, they showed resistance to iprodione, azoxystrobin, and fenpropiate (a lipopeptide produced by *Bacillus subtilis*) (unpublished data). These results indicated that ABC transporters might be involved in the resistance of *F. asiaticum* and *F. graminearum*

to DMI. The search for ABC transporter genes in *F. graminearum* genome showed that there are at least 21 putative ABC transporters existing in this fungal species. Thus, it would be interesting to investigate the roles of these ABC transporters in DMI resistance in FHB pathogens.

Phylogenetic analysis of DNA sequences (16.3-kb) of 13 genes, including α -tubulin, β -tubulin, elongation factor 1 α , histone H3, mating-type locus, reductase, ammonium ligase, phosphate permease, and trichothecene 3-O-acetyltransferase, could differentiate the two genetically related phylogenetic species *F. asiaticum* and *F. graminearum* (32). In this study, we found that *F. asiaticum* could be differentiated from *F. graminearum* based on the DNA sequences of *cyp51A* (Fig. 6). In *Botrytis cinerea*, the HydR1 strains are resistant naturally to the hydroxylanilide fungicide, fenhexamid. Phylogenetic analysis of the DNA sequence of *cyp51* could distinguish HydR1 from non-HydR1 strains (1). The results indicated that *cyp51* genes would be informative markers for analysis of population structure of some phytopathogenic fungi.

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Fig. 7. Relative expression of **A**, *cyp51A* and **B**, *cyp51B* in the demethylation inhibitor-resistant (DMI-R) isolates JS3, JS6, and HN8 and the DMI-sensitive (DMI-S) isolates HB3 and HE-3 of *Fusarium* spp. The relative expression of *cyp51A* and *cyp51B* is the relative amount of each gene mRNA in isolate JS6 without tebuconazole treatment, respectively. Line bars in each column denote standard errors of two experiments.

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