Biological Characteristics and Molecular Mechanisms of Fludioxonil Resistance in *Fusarium graminearum* in China

F. Zhou,^{1,2} D. X. Li,¹ H. Y. Hu,¹ Y. L. Song,² Y. C. Fan,² Y. Y. Guan,¹ P. W. Song,¹ Q. C. Wei,¹ H. F. Yan,³ and C. W. Li^{1,†}

¹ Henan Engineering Research Center of Crop Genome Editing, Henan Institute of Science and Technology, Xinxiang 453003, China

² Henan Engineering Research Center of Biological Fertilizer Developmental and Collaborative Application, Henan Institute of Science and Technology, Xinxiang 453003, China

³ College of Plant Protection, Hebei Agricultural University, Baoding 071001, China

Abstract

Fusarium graminearum is the primary causal agent of Fusarium head blight (FHB) of wheat. The phenylpyrrole fungicide fludioxonil is not currently registered for the management of FHB in China. The current study assessed the fludioxonil sensitivity of a total of 53 *F. graminearum* isolates collected from the six most important wheat-growing provinces of China during 2018 and 2019. The baseline fludioxonil sensitivity distribution indicated that all of the isolates were sensitive, exhibiting a unimodal curve with a mean effective concentration for 50% inhibition value of 0.13 ± 0.12 $\mu\text{g/ml}$ (standard deviation). Five fludioxonil-resistant mutants were subsequently induced by exposure to fludioxonil under laboratory conditions. Ten successive rounds of subculture in the absence of the selection pressure indicated that the mutation was stably inherited. However, the fludioxonil-resistant mutants were found to have reduced pathogenicity, higher glycerol accumulation, and higher osmotic sensitivity than the parental wild-type isolates, indicating that there was a fitness cost associated with fludioxonil resistance. In addition, the study also found a positive cross resistance between fludioxonil, procymidone, and iprodione, but not with other fungicides such

as boscalid, carbendazim, tebuconazole, and fluazinam. Sequence analysis of four candidate target genes (*FgOs1*, *FgOs2*, *FgOs4*, and *FgOs5*) revealed that the HBXT2R mutant contained two point mutations that resulted in amino acid changes at K223T and K415R in its *FgOs1* protein, and one point mutation at residue 520 of its *FgOs5* protein that resulted in a premature stop codon. Similarly, the three other mutants contained point mutations that resulted in changes at the K192R, K293R, and K411R residues of the *FgOs5* protein but none in the *FgOs2* and *FgOs4* genes. However, it is important to point out that the *FgOs2* and *FgOs4* expression of all the fludioxonil-resistant mutants was significantly ($P < 0.05$) downregulated compared with the sensitive isolates (except for the SQ1-2 isolate). It was also found that one of the resistant mutants did not have changes in any of the sequenced target genes, indicating that an alternative mechanism could also lead to fludioxonil resistance.

Keywords: biological characteristics, cross resistance, fludioxonil, *Fusarium graminearum*, resistance mechanism

Fusarium head blight (FHB), which is predominantly caused by *Fusarium graminearum* (teleomorph *Gibberella zeae*), is the most important fungal disease affecting wheat production throughout the world (Haile et al. 2019; Qian et al. 2018; Rawat et al. 2016). FHB not only causes a substantial reduction in grain yield and quality but also results in the production of trichothecene mycotoxins and other secondary metabolites that pose a threat to both human and animal health (Blandino et al. 2012; Chilaka et al. 2017; Goswami and Kistler 2004; Qian et al. 2018; Qiu et al. 2018). Although the development of FHB-resistant wheat cultivars would be a management option (Al-Taweel et al. 2014), the breeding of such varieties has been hampered by a lack of resistant resources. Consequently, the management of FHB relies heavily on the application of chemical fungicides during wheat anthesis to ensure reliable crop production (Liu et al. 2019; Qian et al. 2018; Qiu et al. 2018; Willyerd et al. 2012). Commonly used fungicides include the benzimidazole fungicide carbendazim (Liu et al. 2019; Yuan and Zhou 2005; Zhang

et al. 2009), the cyanoacrylate fungicide phenamacril (Chen et al. 2011; Zhang et al. 2010), the demethylation inhibitors metconazole and tebuconazole (Qian et al. 2018; Tateishi et al. 2010), as well as the strobilurin azoxystrobin (Zhang et al. 2010). However, the extensive and repeated application of these chemicals has led to the frequent emergence of fungicide resistance that could jeopardize their effectiveness, as highlighted by incidences of carbendazim and tebuconazole resistance (Qian et al. 2018; Zhang et al. 2010).

The phenylpyrrole fungicide fludioxonil, which has broad-spectrum activity against both basidiomycete and ascomycete pathogens, could provide a novel opportunity to control FHB (Brandhorst et al. 2019; Furukawa et al. 2012; Hu et al. 2019; Yoshimi et al. 2004). It is widely believed that the target site of fludioxonil is a histidine kinase (HK) related to the high-osmolarity glycerol (HOG1) stress response signal transduction pathway, although the precise mode of action has yet to be characterized fully (Brandhorst et al. 2019; Furukawa et al. 2012; Yoshimi et al. 2004). This signaling pathway is involved in many fungal processes, which perhaps explains why fludioxonil exhibits such a broad spectrum of activity against such a wide range of fungal pathogens (Brandhorst et al. 2019; Koch and Leadbeater 1992; Yoshimi et al. 2004). Indeed, fludioxonil has been used extensively against multiple plant pathogens in the past 30 years. In China, fludioxonil has been used control diseases of wheat, rice, cotton, and peanut for many years (Qiu et al. 2018; Sang et al. 2018). Although fludioxonil has not been widely applied in the field to control FHB, wheat seed are routinely treated with the fludioxonil containing formulation Beret Gold (Syngenta) (active ingredient: fludioxonil at 24.3 $\mu\text{g/ml}$) before sowing to prevent soilborne diseases (Bailey et al. 2005; Qiu et al. 2018). Given such a history of heavy use, it is not surprising that fludioxonil-resistant isolates of *F. graminearum* have been detected in corn and soybean seedling samples (Broders et al. 2007), and a recent report has detailed the emergence of fludioxonil-resistant isolates of *F. asiaticum*

[†]Corresponding author: C. W. Li; lichengweiwau@hotmail.com

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in the wheat fields of China (Qiu et al. 2018). Although there have been no similar incidents of fludioxonil resistance in *F. graminearum* isolates in China, the emergence of resistance in the closely related species *F. asiaticum* has raised concerns that resistance could also arise in *F. graminearum* (Qiu et al. 2018). However, previous studies have shown that fludioxonil-resistant isolates often have reduced fitness and exhibit increased sensitivity to osmotic stress, which is consistent with observations that fludioxonil can inhibit the HOG1 cascade of the mitogen-activated protein (MAP) kinase signaling pathway (Lew 2010; Qiu et al. 2018; Sang et al. 2018). Indeed, studies in *Botrytis cinerea* have revealed that highly resistant laboratory mutants have altered sequences in the ATPase domain of the C terminal of an upstream HK, while field populations exhibit mutations predominantly distributed among the histidine kinases, adenylyl cyclases, methyl accepting chemotaxis proteins, and phosphatases (HAMP) domains of the N terminal in HK, a motif which is necessary for the recognition of stimulating compounds (Qiu et al. 2018; Ren et al. 2016; Sang et al. 2018). For example, mutations within the HAMP domains in the N-terminal region of the group III HK OS-1 in laboratory mutants have been shown to confer a high level of fludioxonil resistance (Duan et al. 2014a; Fillinger et al. 2012). In addition, mutant *Os1* alleles in *Neurospora crassa* have been reported to have either single or multiple mutations within the six-tandem-repeat motif (Miller et al. 2002), while studies on mutant strains of *Cochliobolus heterostrophus* and *N. crassa* have also indicated that resistance to dicarboximide and phenylpyrrole fungicides is influenced by the structure of the amino acid repeat region (Yoshimi et al. 2004). However, some plant pathogens are known to have multiple *HK* genes, indicating that mutations in other *Os*-like genes may be possible causes of fludioxonil resistance (Catlett et al. 2003; Qiu et al. 2018).

Although fludioxonil resistance has been reported in field isolates of *F. asiaticum* (Qiu et al. 2018), and low levels of resistance have been detected in many plant pathogens (Avenot and Michailides 2015; Dry et al. 2004; Duan et al. 2014a; Han et al. 2017; Jung et al. 2012; Li and Xiao 2008; Peters et al. 2008; Ren et al. 2016; Tückmantel et al. 2011; Wu et al. 2015), the precise mechanism of fludioxonil resistance has yet to be characterized in detail. Further research is required to elucidate the biological characteristics and molecular mechanism of fludioxonil resistance. The specific objectives of the current study were to (i) establish the baseline sensitivity of *F. graminearum* to fludioxonil; (ii) compare the fitness parameters and physiological characteristics of sensitive and fludioxonil-resistant isolates; (iii) determine whether there are any patterns of cross resistance between fludioxonil and other widely used fungicides, including procymidone, iprodione, boscalid, carbendazim, tebuconazole, and fluazinam; and (iv) identify potential mechanism of fludioxonil resistance in *F. graminearum*.

Materials and Methods

Isolates of *F. graminearum*, media, and mycelia preparation. Infected wheat samples were collected from six mainly wheat-growing provinces of China (Henan, Hebei, Anhui, Jiangsu, Sichuan, and Guizhou Provinces) in summer 2018 and 2019 (Supplementary Table S1). The samples were collected from wheat grains exhibiting typical symptoms of FHB, and individual *F. graminearum* isolates were purified by single-spore isolation according to the protocol of a previous study (Qiu et al. 2018). The fungal cultures used in the study were routinely maintained on potato dextrose agar (PDA; potato at 200.0 g/liter, agar at 20.0 g/liter, and dextrose at 20.0 g/liter), and their spores suspended in a 20% sterile-glycerol solution for long-term storage at -20°C .

Five fludioxonil-sensitive isolates, including 2XZ-4, HBXT2, CM2, SQ1-2, and YN1-3, which had effective concentration for 50% inhibition (EC_{50}) values of 0.06, 0.01, 0.05, and 0.04 $\mu\text{g/ml}$, respectively, were selected for further study. Repeated fludioxonil exposure resulted in the selection of the five highly resistant laboratory mutants 2XZ-4R, HBXT2R, CM2R, SQ1-2R, and YN1-3R with EC_{50} values of 41.36, 48.75, 68.45, 101.78, and 73.21 $\mu\text{g/ml}$, respectively.

The cultures used for the intracellular glycerol accumulation assay and genomic DNA extraction were prepared in a conical flask

according to the protocols of previous studies (Qiu and Shi 2014; Qiu et al. 2018). Each isolate was incubated in potato dextrose broth (PDB) medium (Beijing Aoboxing Bio-Tech Co. Ltd.) for 72 h at 23°C with shaking (130 rpm). The mycelium was then harvested and washed three times in PDB before being flash-frozen in liquid nitrogen. In the case of the glycerol assay, an additional step involving the transfer of the harvested mycelium to fresh PDB amended with 0.1 μM fludioxonil and incubation at 23°C for a further 5 h was included, before the mycelium was harvested a second time, washed, and flash-frozen in liquid nitrogen.

Fungicides. Technical-grade fungicides used in the study, including 95.3% iprodione (Heyi Agrochemical Co. Ltd.), 98.0% procymidone (Heyi Agrochemical Co. Ltd.), 97.0% boscalid (Zhejiang Heben Pesticide & Chemicals Co. Ltd.), 96.2% tebuconazole (Sheyang Huanghai Pesticide Chemical Co. Ltd.), and 96.0% fluazinam (Hubei Jianyuan Chemical Co. Ltd.), were dissolved in acetone to produce stock solutions of 10,000 $\mu\text{g/ml}$, while the 98.1% carbendazim (Haili Guixi Chemical Co. Ltd.) was dissolved in hydrochloric acid at 0.1 mol/liter (Supplementary Table S2). The stock solutions were stored at 4°C for no longer than 2 weeks before being used to prepare the serial dilutions used in the experiments. Mycelial growth assays were used to confirm that the solvents had no effect on the growth of *F. graminearum* at the range of concentrations tested (data not shown).

Baseline sensitivity of *F. graminearum* to fludioxonil. In total, 53 *F. graminearum* isolates were used to determine baseline EC_{50} values for fludioxonil using the mycelial growth assay described in a previous study (Zhou et al. 2017). Briefly, fresh mycelium plugs (5 mm in diameter) from 3-day-old cultures were transferred to PDA containing fludioxonil at 0, 0.001, 0.005, 0.025, 0.125, 0.625, 3.125, and 15.625 $\mu\text{g/ml}$. The radial growth of the resulting mycelium was measured after 72 h of incubation at 23°C . The percent inhibition was then calculated using the following formula: $1 - (\text{radial growth on fungicide-amended PDA medium} / \text{radial growth on PDA medium}) \times 100$, while the EC_{50} values were estimated by linear regression of $\log_{10}$ fungicide concentrations versus the probit value of percent growth inhibition. Each isolate was represented by three separate plates and the entire experiment was performed twice.

Heritable stability of fludioxonil resistance. The hereditary stability of the five fludioxonil-resistant *F. graminearum* mutants (2XZ-4R, HBXT2R, CM2R, SQ1-2R, and YN1-3R) was assessed with a modified version of the protocol used by Hu et al. (2019). Each mutant was subjected to 10 successive rounds of subculture on fludioxonil-free PDA before their fludioxonil EC_{50} values were reevaluated at the following concentrations: 0, 1, 5, 50, 100, 200, 300, and 600 $\mu\text{g/ml}$. The resistance factor (RF) was then calculated by comparing the EC_{50} values of the resistant mutants to those of their parental isolates according to the following formula: $\text{RF} = \text{EC}_{50}$ of the mutant / EC_{50} of parent isolate. The stability of resistance itself was estimated from the change in EC_{50} values calculated for the 1st and 10th subculture. Each isolate was represented by three replicate plates and the entire experiment was performed twice.

Biological characteristics of fludioxonil-resistant *F. graminearum* mutants. *Mycelial growth.* The mycelial growth of four sensitive wild-type *F. graminearum* isolates (2XZ-4, HBXT2, CM2, and SQ1-2) and five laboratory fludioxonil-resistant mutants (2XZ-4R, HBXT2R, CM2R, SQ1-2R, and YN1-3R) was evaluated as previously described (Zhou et al., 2020). Briefly, mycelial plugs (5 mm in diameter) were taken from the edge of 2-day-old colonies and transferred to fresh PDA plates that were then incubated at 23°C with a 12-h photoperiod. The resulting colonies were observed daily and the diameter of each measured at 24, 48, and 72h postinoculation. Each isolate was represented by six separate plates and the entire experiment was performed once.

Sporulation. The rate of sporulation of the sensitive and resistant isolates was assessed on PDA using the method of Duan et al. (2018), with a few modifications. The test colonies were initially established by transferring 5-mm mycelial plugs from 2-day-old PDA cultures to flasks containing 30 ml of mung bean broth. After

3 days of incubation at 23°C with shaking (130 rpm), the resulting spores were harvested and counted using a hemocytometer (Shanghai Qiujiing Biochemical Reagent Instrument Co., Ltd.). Each isolate was represented by at least three replicate flasks, and the entire experiment was performed twice.

Sensitivity to osmotic pressure. The response of the resistant and sensitive isolates to osmotic stress was performed according to the protocol of a previous study (Qiu et al. 2018), with a few modifications. Mycelial plugs (5 mm) from 2-day-old PDA cultures were transferred to fresh PDA plates amended with 0.5 M KCl, 0.5 M glucose, 0.5 M MgCl₂, or 0.5 M mannitol. Identical amendment-free cultures were used as the control. The diameters of the colonies were measured after 48 h of incubation at 23°C with a 12-h photoperiod, and the percent inhibition of mycelial growth was calculated according to the formula detailed in the previous study (Qiu et al. 2018). Each treatment was represented by six replicate plates, and the entire experiment performed twice.

Pathogenicity on wheat. The pathogenicity of the resistant and sensitive isolates was assessed on wheat seedlings using the in vitro assay described in previous studies (Duan et al. 2018; Liu et al. 2013; Yang et al. 2018; Zhang et al. 2017), with a few minor modifications. Wheat coleoptiles from 3-day-old seedlings (cultivar Bainong 207) were cut, and their exposed surfaces were inoculated with 2-μl spore suspensions (10⁵ spores/ml), or water in the case of the negative controls. The seedlings were then maintained at 23°C with 95% relative humidity and a 16-h photoperiod, and the length of the infection lesions was determined at 15 days postinoculation. Each isolate was represented by at least 15 replicate coleoptiles, and the entire experiment was performed twice.

Fig. 1. Frequency distribution of fludioxonil effective concentration for 50% inhibition (EC_{50}) values for 53 *Fusarium graminearum* isolates collected from the six primary wheat-producing provinces of China during the summers of 2018 and 2019.

Fig. 2. Heritable stability of fludioxonil resistance in five *Fusarium graminearum* mutants assessed by the variation in fludioxonil effective concentration for 50% inhibition (EC_{50}) values during 10 successive rounds of subculture.

Determination of glycerol content. The glycerol accumulation of the resistant and sensitive isolates was determined using the protocol applied in two previous studies (Zhang et al. 2017; Zhou et al. in press), with a few modifications. *F. graminearum* cultures were first established in 75 ml of PDB media by inoculation with a single mycelial plug (5 mm). The test cultures were then incubated for 3 days at 23°C with shaking (150 rpm) before the mycelium from each was harvested, washed three times in PDB, and transferred to fresh media in the absence or presence of fludioxonil at 0.1 μg/ml. After a further 5 h of incubation, the mycelia were reharvested, washed, and ground under liquid nitrogen using a grinder, before being transferred to the glycerol extraction buffer. The glycerol content was then measured using a commercial glycerol assay kit (Applygen, Beijing, China) according to the instructions of the manufacturer. Each isolate was represented by at least three replicate samples, and the entire experiment was performed twice.

Cloning and sequencing of the *FgOs1*, *FgOs2*, *FgOs4*, and *FgOs5* genes. Fresh mycelia were collected from 200-ml PDB cultures, and their genomic DNA was extracted according to the protocol of De Miccolis Angelini et al. (2014). Primer sets developed in a previous study (Qiu et al. 2018) were then used to amplify the full-length sequence of each candidate gene. The primers had the following sequences: *FgOs1F1*/*FgOs1R1*, ACCCACCCGTTCAAACCTACAC/ATCTCGCTGATGCCTCTAC; *FgOs1F2*/*FgOs1R2*, CAAGCCTGAACACGAACAAC/AGCAACGAATAACAGAGCC; *FgOs2F*/*FgOs2R*, ACCACACCTATCAAACCACTGC/TTCCCTTATCTCCC CAACG; *FgOs4F1*/*FgOs4R1*, GCAGCCACAGCAAGACGAA/CGG GGACGCAATCACATAGA; *FgOs4F2*/*FgOs4R2*, CGACTGAAATGAGCAAACGC/AGAAGAAAGAGGAAGTGAAAG; and *FgOs5F*/*FgOs5R*, TTACCGTCCCTGGGATTCTAC/CCTGCCCTTCCTTATCTGTCTT. The PCRs were performed using 50-μl reaction mixtures containing 25 μl of 2× ES Taq Master Mix, 1.5 μl of template DNA, 2 μl of each primer, and 21.5 μl of double-distilled H₂O according to the PCR kit obtained from CoWin Biosciences, and processed using a 96-well thermal cycler (Applied Biosystems, Thermo Fisher Scientific) with the following program: an initial denaturation at 94°C for 2 min; followed by 35 cycles of 94°C for 30 s, 57°C for 30 s, and 72°C for 2.5 min; and a final extension at 72°C for 10 min. The resulting PCR products were then purified and cloned into the PMD19-T vector using a cloning kit (TaKaRa) and sequenced commercially (Wuhan Genecreate Biotechnology Co. Ltd). The predicted amino acid sequences were then aligned using the DNAMAN software package (ver.8.0; Lynnon Biosoft), and amino acid differences between resistant and sensitive isolates were determined as described previously (Gong et al. 2018; Zhou et al. 2017, 2020).

Comparison of expression levels of *FgOs2* and *FgOs4* genes in fludioxonil-resistant mutants of *F. graminearum*. Total RNA was extracted from mycelia using a fungal RNA kit (Omega Bio-Tek) according to the manufacturer's instructions, and used as a template for the amplification of *FgOs2* and *FgOs4* genes (about 400 bp) using the following primer pairs: *FgOs2* qPCR F1/*FgOs2* qPCR R1, *FgOs4* qPCR F1/*FgOs4* qPCR R1, and *Fg β-tubulin* F1/*Fg β-tubulin* F1, which had the sequences CATGCGTGGACTCAAGTACG/GAGCTCGGTGATGATGGAGA, ACAAAGCTTGCCCTTGACTG/CCTCGTTCGTTTCATCATTCGG, and GCGAGGTTGAGAACTGTGAC/GCGAGGTTGAGAACTGTGAC, respectively. First-strand cDNA was prepared with the PrimeScript RT reagent kit (TaKaRa). Real-time PCR amplifications were performed on applied biosystems of QuantStudio 6 Flex PCR detection systems (Thermo Fisher) using SYBR Green I fluorescent dye detection. The relative quantities were calculated using the described methods previously (Duan et al. 2018). The *β-tubulin* as a reference gene was used in this study, and three biological replicates for each mutant were used to calculate the mean and standard error.

Cross resistance between fludioxonil and other fungicides. The potential cross resistance between fludioxonil and six commonly used fungicides, including carbendazim, iprodione, procymidone, boscalid, tebuconazole, and fluazinam, was assessed using the mycelial growth assay approach, as described previously (Zhou et al. 2014, in press). The same protocol used to determine the fludioxonil EC_{50} values described above was used to determine similar values

for each of the different fungicides in each of the fludioxonil-resistant and -sensitive isolates. At least seven concentrations were used for each fungicide: 0, 1.5625, 3.125, 6.25, 12.5, 25, 50, and 100 $\mu\text{g/ml}$ in the case of fludioxonil, iprodione, and procymidone; and 0, 0.0625, 0.125, 0.025, 0.05, 0.10, 0.20, 0.40, 0.80, and 1.60 $\mu\text{g/ml}$ with carbendazim, boscalid, fluazinam, and tebuconazole. Each treatment was represented by at least three replicate plates, and the entire experiment was conducted twice.

Data analysis. The data collected during the current study were first evaluated by analysis of variance using SPSS software (ver. 17.0; SPSS Inc.). Fisher's least significant difference test ($\alpha = 0.05$ and $\alpha = 0.01$) was then used to determine the statistical differences between different treatments. The DNA sequencing data were analyzed using the software DNAMAN (version 6.0; Lynnon Biosoft).

Results

Baseline sensitivity of *F. graminearum* to fludioxonil. The current study found that none of the 53 *F. graminearum* isolates collected from wheat-growing provinces of China were able to grow on PDA amended with fludioxonil at 5 mg/ml. The frequency distribution of their EC_{50} values conformed to a unimodal distribution (Fig. 1), with values ranging from 0.01 to 0.45 $\mu\text{g/ml}$ and a mean of 0.13 ± 0.12 $\mu\text{g/ml}$ ($\pm$ standard deviation [SD]). All 53 wild-type isolates were completely inhibited by a fludioxonil concentration of 5.0 $\mu\text{g/ml}$.

Heritable stability, biological characteristics, and osmotic sensitivity associated with fludioxonil resistance. After 10 successive rounds of subculturing, the EC_{50} values of the five fludioxonil-resistant mutants had not changed significantly (Fig. 2; Supplementary

Table 1. Mycelial growth, sporulation, and pathogenicity of five fludioxonil-resistant mutants and four sensitive isolates of *Fusarium graminearum*^y

Isolate (EC_{50}) ^w	Mycelial growth (cm) $\pm$ SE ^x			Spores/ml ^y	Pathogenicity ^z
	24 h	48 h	72 h		
2XZ-4 (0.06)	1.18 $\pm$ 0.05 a A	3.13 $\pm$ 0.15 a A	5.73 $\pm$ 0.03 c B	10.17 $\pm$ 1.19 a A	2.63 $\pm$ 0.07 d DEF
HBXT2 (0.01)	1.75 $\pm$ 0.07 bc BC	3.23 $\pm$ 0.05 a A	4.78 $\pm$ 0.21 a A	9.33 $\pm$ 0.84 a A	2.37 $\pm$ 0.36 cd DEF
CM2 (0.05)	2.05 $\pm$ 0.10 d C	4.55 $\pm$ 0.07 c C	5.70 $\pm$ 0.00 bc B	25.34 $\pm$ 0.38 bc AB	3.50 $\pm$ 0.26 e F
SQ1-2 (0.04)	1.90 $\pm$ 0.10 cd BC	4.23 $\pm$ 0.09 bc BC	5.70 $\pm$ 0.00 bc B	20.50 $\pm$ 3.71 ab AB	2.73 $\pm$ 0.15 de EF
2XZ-4R (41.36)	1.60 $\pm$ 0.10 bc B	3.98 $\pm$ 0.08 b B	5.55 $\pm$ 0.05 bc B	20.67 $\pm$ 1.80 ab AB	0.00 $\pm$ 0.00 a A
HBXT2R (48.75)	1.50 $\pm$ 0.04 b AB	3.35 $\pm$ 0.19 a A	5.48 $\pm$ 0.17 b B	38.33 $\pm$ 6.29 cd BC	1.67 $\pm$ 0.20 bc CDE
CM2R (68.45)	1.65 $\pm$ 0.10 bc BC	4.15 $\pm$ 0.16 b BC	5.58 $\pm$ 0.05 bc B	40.17 $\pm$ 8.09 d C	1.50 $\pm$ 0.29 b BCD
SQ1-2R (101.78)	1.78 $\pm$ 0.16 bcd BC	4.25 $\pm$ 0.07 bc BC	5.70 $\pm$ 0.00 bc B	34.17 $\pm$ 4.30 bc BC	0.83 $\pm$ 0.15 ab ABC
YN1-3R (73.21)	1.88 $\pm$ 0.11 cd BC	3.98 $\pm$ 0.10 b B	5.73 $\pm$ 0.03 c B	20.33 $\pm$ 2.23 ab AB	0.47 $\pm$ 0.15 a AB

^v The same letter in the same column indicates that the difference was not significant according to Fisher's least significant difference test ($P = 0.05$ or $P = 0.01$) in SPSS. Different letters in the same column indicate significant differences according to Fisher's least significant difference test ($P = 0.05$ or $P = 0.01$).

^w EC_{50} = effective concentration for 50% inhibition values for fludioxonil.

^x SE = standard error.

^y Sporulation.

^z Pathogenicity on wheat coleoptiles.

Fig. 3. Mycelial growth of five fludioxonil-resistant mutants and four sensitive isolates of *Fusarium graminearum* in response to osmotic stress induced by **A**, 0.5 M KCl; **B**, 0.5 M glucose; **C**, 0.5 M mannitol; and **D**, 0.5 M MgCl_2 . Error bars indicate the standard error from two separate experiments. Different letters above columns indicate significant differences according to Fisher's least significant difference test ($\alpha = 0.05$).

Table S3), indicating that the resistance was stable. Investigation of their biological characteristics indicated that the mycelial growth of the fludioxonil-resistant mutants was little affected but that the sporulation of four mutants (HBXT2R, CM2R, SQ1-2R, and YN1-3R) was significantly increased relative to the sensitive wild-type isolates (Table 1). However, the pathogenicity of all the fludioxonil-resistant isolates was significantly reduced ($P < 0.05$), with the mutant 2XZ-4R completely losing its ability to infect wheat coleoptiles (Table 1). In addition, it was also found that all of the fludioxonil-resistant mutants had significantly ($P < 0.05$) reduced growth rates in response to osmotic stress as assessed on PDA amended with 0.5 M KCl (Fig. 3A), 0.5 M glucose (Fig. 3B), 0.5 M mannitol (Fig. 3C), and 0.5 M MgCl₂ (Fig. 3D). Taken together, these results indicate that the fludioxonil-resistant mutants had significantly reduced fitness with regard to both pathogenicity and osmotic stress.

Glycerol accumulation in fludioxonil-resistant mutants of *F. graminearum*. The glycerol content of all the fludioxonil-resistant mutants was significantly increased relative to the sensitive wild-type isolates when grown on PDB in the absence of fludioxonil (Fig. 4). However, with the addition of fludioxonil at 0.1 µg/ml, this trend was reversed, with the sensitive isolates 2XZ-4, HBXT2, CM2, and SQ1-2 generally having a higher glycerol content, increased by 3.35-, 1.79-, 1.96-, and 2.09-fold, respectively. In contrast, the fludioxonil treatment had a much less dramatic, although still significant, effect on the fludioxonil-resistant mutants, increasing by just 1.30-, 1.09-, 1.06-, 1.27-, and 1.07-fold for isolates 2XZ-4R, HBXT2R, CM2R, SQ1-2R, and YN1-3R, respectively (Fig. 4).

Fig. 4. Glycerol content of five resistant mutants and four sensitive isolates of *Fusarium graminearum* in response to fludioxonil treatment. Error bars indicate the standard error from two separate experiments. Different letters above columns indicate significant differences according to Fisher's least significant difference test ($\alpha = 0.05$).

Cross resistance with other fungicides in fludioxonil-resistant mutants of *F. graminearum*. The current study found a positive cross resistance between fludioxonil and the dicarboximide fungicides procymidone and iprodione. This is not surprising because the dicarboximides are known to share similar modes of action with fludioxonil and also target the HOG-MAP kinase signal transduction pathway. However, no cross resistance was found between fludioxonil and any of the other fungicides tested, including carbendazim, boscalid, tebuconazole, and fluazinam (Table 2).

Sequence analysis of the *FgOs1*, *FgOs2*, *FgOs4*, and *FgOs5* genes in fludioxonil-resistant mutants of *F. graminearum*. The open reading frame sequences of the four *F. graminearum* *Os* genes (*FgOs1*, *FgOs2*, *FgOs4*, and *FgOs5*), which have previously been associated with fludioxonil resistance in *F. asiaticum*, were successfully cloned from the five fludioxonil-resistant mutants and four wild-type isolates. The predicted protein sequences identified several amino acid changes (Supplementary Table S4). HBXT2R had two point mutations, K223T and K415R, in its *FgOs1* gene, as well as one in its *FgOs5* gene that resulted in a premature stop codon at amino acid 520. Three of the other mutants, 2XZ-4R, CM2R, and SQ1-2R, had single mutations in their *FgOs5* gene, which resulted in the amino acid changes of K192R, K293R, and K411R, respectively. However, it was interesting to note that the fifth mutant, YN1-3R, had no mutations in any of the sequenced target genes, indicating that an alternative resistant mechanism was responsible for its reduced sensitivity to fludioxonil (Table 3). No amino acid changes were detected in the predicted *FgOs2* and *FgOs4* sequences of any of the mutants tested.

Comparison of expression levels of *FgOs2* and *FgOs4* genes in fludioxonil-resistant mutants of *F. graminearum*. In this study, the expression levels of *FgOs2* and *FgOs4* in all isolates were assessed. Results showed that *FgOs2* and *FgOs4* expressions of all the fludioxonil-resistant mutants significantly ($P < 0.05$) downregulated compared with the sensitive isolates (except for the SQ1-2 isolates) when grown in PDB media. Meanwhile, similar results were also observed when the isolates were treated with fludioxonil at 0.1 µg/ml (Fig. 5). These results indicated that the candidate target genes *FgOs2* and *FgOs4* maybe also related to the fludioxonil resistance.

Discussion

The FHB caused by *F. graminearum* is an important fungal disease affecting wheat production throughout the world (Haile et al. 2019; Qian et al. 2018; Rawat et al. 2016). Previous research has shown that several fungicides, including carbendazim, tebuconazole and phenamacril, are at high risk for the development of resistance, jeopardizing their capacity to control FHB in China (Chen et al. 2011; Duan et al. 2014b; Liu et al. 2019; Qian et al. 2018). However, inappropriate use or overuse can lead to an increased selection pressure on pathogens that results in fungicide resistance (Brent and Hollomon 1998). The current study evaluated 53 *F. graminearum* isolates collected from the six primary wheat-producing provinces of China in summer 2018 and 2019, and found that all of these wild-type isolates remained sensitive to fludioxonil, having a baseline fludioxonil sensitivity with a mean EC₅₀ value of 0.13 ± 0.12 µg/ml (SD). Further investigation of five fludioxonil-resistant mutants produced by exposure to fludioxonil under laboratory

Table 2. Cross resistance between fludioxonil and six commonly used fungicides in *Fusarium graminearum*^z

Fungicides	Sensitive isolates				Fludioxonil-resistant mutants				
	2XZ-4	HBXT2	CM2	SQ1-2	2XZ-4R	HBXT2R	CM2R	SQ1-2R	YN1-3R
Fludioxonil	0.07 ^z	0.07	0.03	0.03	41.36	48.75	68.45	101.78	73.21
Procymidone	0.35	0.46	0.42	0.77	36.88	>50.00	>50.00	78.65	>50.00
Iprodione	0.87	1.77	0.72	1.36	0.99	96.78	68.96	>50.00	>50.00
Boscalid	0.10	0.14	0.08	0.01	0.16	0.22	0.07	0.31	0.26
Carbendazim	0.730	0.54	0.63	0.35	0.62	0.46	0.33	0.71	0.29
Tebuconazole	0.19	0.03	0.08	0.12	0.08	0.07	0.21	0.08	0.11
Fluazinam	0.10	0.04	0.13	0.01	0.09	0.01	0.16	0.05	0.03

^z Effective concentration for 50% inhibition values.

conditions, as well as four sensitive wild-type isolates, found that the resistant mutants had reduced fitness with regard to both pathogenicity and osmotic stress. Furthermore, the mutants exhibited higher levels of glycerol accumulation, all of which are consistent with the findings of other studies investigating the ascomycete pathogen *B. cinerea* (Gong et al. 2018; Ren et al. 2016; Zhou et al., 2020). Similar findings have also been reported in studies of *F. asiaticum* in China (Qiu et al. 2018). It is also interesting to note that dimethachlone-resistant isolates of *Sclerotinia sclerotiorum* and fenhexamid-resistant mutants of *B. cinerea* also exhibit similar patterns of fitness and virulence (Zhou et al. 2014, 2017).

Although fludioxonil has strong activity against a wide range of plant pathogens, and has extensively been used for the control of numerous diseases over the last 30 years, the mode of action of fludioxonil has still not been characterized in detail (Brandhorst et al. 2019; Koch and Leadbeater 1992; Qiu et al. 2018; Sang et al. 2018). However, many studies have indicated that the protein target of fludioxonil is an HK associated with the HOG1 cascade MAP kinase signaling pathway and that fludioxonil-resistant mutants have increased sensitivity to osmotic stress (Brandhorst et al. 2019; Gong et al. 2018; Qiu et al. 2018; Ren et al. 2016). The latter observation is of great interest because it is well known that the fitness of resistant mutants is an extremely important parameter regarding the risk for development of fungicide resistance (Brent and Hollomon 2007). Cross resistance between different fungicides is also an important risk factor and, although there were insufficient studies to determine the full extent of cross resistance between fludioxonil and other fungicides, the results from investigations focusing on *S. homoeocarpa* and *B. cinerea* have indicated that fludioxonil resistance can lead to reduced sensitivity to dicarboximide fungicides such as iprodione, vinclozolin, and tolclofos-methyl but not to other fungicides such as propiconazole, fenpropimorph, cyprodinil, tridemorph, epoxiconazole, spiroxamine, fluazinam, and chlorothalonil. Conversely, a study of fludioxonil-resistant mutants of *Aspergillus carbonarius* actually found an increased sensitivity to the strobilurin pyraclostrobin (Malandrakis et al. 2013). The results of the current study reconfirmed these prior observations, finding a positive cross resistance between fludioxonil and the dicarboximide fungicides procymidone and iprodione but none with any of the other fungicides tested, including boscalid, carbendazim, tebuconazole, and fluazinam (Table 2). These results are not particularly surprising because it is known that fludioxonil and dicarboximides share similar mode of action. Taken together, these results indicated that the inclusion of fludioxonil in conjunction with nondicarboximide fungicides within an integrated pest management (IPM) program could help to minimize the risk of fludioxonil resistance development.

Several reports have suggested that mutations in the Os-1 HK upstream of the HOG1 MAP kinase signaling pathway can contribute to fludioxonil resistance in *Alternaria brassicicola* and *B. cinerea*, especially mutations in the N-terminal region of the *Os1* gene (Avenot and Simoneau 2005; Ren et al. 2016). Similarly, several amino acid mutations in the *FgOs1* of *F. asiaticum* have been linked to fludioxonil resistance, as well as changes in its *FgOs4* and *FgOs5* genes (Qiu et al. 2018). The molecular analysis conducted in the current study found evidence of similar mutations in *F. graminearum*. For example, the *FgOs1* gene of the fludioxonil-resistant mutant HBXT2R contained two point mutations that resulted in amino acid changes at K223T and K415R in addition to a premature stop codon in its *FgOs5* gene, while the resistant mutants 2XZ-4R, CM2R, and SQ1-2R had single point mutations in their *FgOs5* genes that resulted in changes at K192R, K293R, and K411R, respectively. These point mutations were different from the previous reports (Supplementary Table S4) of the fludioxonil resistance in the MAP kinase protein in *F. graminearum* and *F. asiaticum*. However, none of the resistant mutants were found to have mutations in their *FgOs2* and *FgOs4* genes, and it is interesting to note that none of the mutations found in the *FgOs1* and *FgOs5* genes were the same as those documented in the *F. asiaticum* study (Qiu et al. 2018). However, the *FgOs2* and *FgOs4* expression of all of the fludioxonil-resistant mutants was significantly ($P < 0.05$) downregulated compared with the sensitive

isolates (except for the SQ1-2 isolate), indicating that the candidate target genes *FgOs2* and *FgOs4* may also be related to the fludioxonil resistance, which was similar to dimethachlone resistance to *S. sclerotiorum* (Li et al. 2017). It is also noteworthy that the fludioxonil-resistant mutant YN1-3R did not have mutations in any of its candidate target genes. Taken together, these findings indicated that the mechanism of fludioxonil resistance in *F. graminearum* could be much more complex than previously thought, and could involve alternative resistance mechanisms. Further molecular-genetic analysis is required to completely characterize the mechanism of fludioxonil resistance in this species.

In summary, the current study found that no evidence of field resistance to fludioxonil in any of the *F. graminearum* samples collected from the six primary wheat-producing provinces of China. Analysis of the biological characteristics of five laboratory-induced fludioxonil-resistant mutants found significantly reduced fitness with regard to both osmotic sensitivity and pathogenicity. Considering the

Table 3. Mutations in the *FgOs1*, *FgOs2*, *FgOs4*, and *FgOs5* protein sequence of fludioxonil-resistant mutants of *Fusarium graminearum*^y

Location				
Genes	Types ^z	Gene numbers	Mutants	Mutation type
<i>FgOs1</i>	Histidine kinase	FGSG_07118	HBXT2R	K223T, K415R
<i>FgOs5</i>	MAPKK	FGSG_08691	2XZ-4R	K192R
<i>FgOs5</i>	ND	FGSG_08691	HBXT2R	520 stop
<i>FgOs5</i>	ND	FGSG_08691	CM2R	K293R
<i>FgOs5</i>	ND	FGSG_08691	SQ1-2R	K411R
<i>FgOs2</i>	MAPK	FGSG_09612	ND	ND
<i>FgOs4</i>	MAPKKK	FGSG_00408	ND	ND

^y ND indicates no data available.

^z MAPKK = mitogen-activated protein (MAP) kinase kinase, MAPK = MAP kinase, and MAPKKK = MAP kinase kinase kinase.

Fig. 5. Expression levels of **A**, *FgOs2* and **B**, *FgOs4* genes in fludioxonil-resistant mutants and sensitive isolates in *Fusarium graminearum*. CK = control. Values are means ($\pm$ standard error) of two independent repeats. Error bars denote the standard error of the mean according to Fisher's least significant difference test ($\alpha = 0.05$).

critical role of chemical control in the management of FHB, it is important that measures are taken to manage the risk of fungicide resistance development. The observation that the fludioxonil-resistant mutants assessed in the current study exhibited no cross resistance with boscalid, carbendazim, tebuconazole, and fluzinam indicates that the combined or alternated application of fludioxonil with these fungicides within an IPM program could provide effective and sustained control of FHB in wheat production. However, despite these precautions, the discovery of the YN1-3R mutant, which appears to have a novel mechanism of resistance, demonstrates that it is important to continue monitoring fludioxonil resistance in the field, and that further research is still required to completely characterize the mechanisms of resistance in *F. graminearum* and guarantee the future safety of wheat production in China.

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