



## Premature termination codon mutations in osmosensor-like histidine kinase *FgOs1* endow *Fusarium graminearum* with fludioxonil resistance

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### ABSTRACT

*Fusarium graminearum* is a devastating disease in cereal production, causing a loss in grain production. Fludioxonil, a phenylpyrrole fungicide, has been registered for disease management of many crops. However, the resistance mechanism of *F. graminearum* to fludioxonil has not been systematically analyzed. This study elucidates the molecular basis of fludioxonil resistance in *F. graminearum* by demonstrating that premature termination codon mutations in the osmosensor-like histidine kinase gene *FgOs1* are causative factors. Two independent mutations were identified in fludioxonil-resistant mutants, leading to truncated *FgOs1* proteins: a *FgOs1*<sup>Q140STOP</sup> mutation locating at N-terminal and an *FgOs1*<sup>R1183STOP</sup> mutation locating at the REC signaling domain. Homologous gene replacement and complementation assays confirmed that these mutations specifically confer high-level resistance to fludioxonil (resistance factor > 1000) without cross-resistance to tebuconazole, phenamacril, or carbendazim. Notably, *FgOs1*-mutated strains exhibited heightened sensitivity to osmotic and metal ion stresses, suggesting that the premature termination codon-induced protein truncation impairs osmoregulation and ion homeostasis pathways. Phylogenetic analysis revealed that the mutated residues are highly conserved across diverse fungal species, underscoring their functional importance. These findings uncover a previously unrecognized role of *FgOs1* in antifungal resistance and provide critical targets for developing innovative strategies to manage fludioxonil resistance in *F. graminearum*.

### 1. Introduction

The pathogenic ascomycete *Fusarium graminearum* is one of the major causal agents of maize stalk rot and ear rot (Dean et al., 2012). *Fusarium* diseases not only cause severe yield losses, but the pathogens produce mycotoxins in infected maize that pose serious threats to human and animal health (Chen et al., 2019). In the last decade, maize stalk rot and ear rot have spread rapidly to other maize-growing areas due to climate change, intensified maize cultivation, and the incorporation of straw (Zhang et al., 2012; Sun et al., 2020). After infecting corn, *F. graminearum* can cause browning and necrosis of the vascular bundles in the stalks, impairing the transport of water and nutrients, leading to plant lodging or wilting. At the same time, it infects the ears, resulting in grain rot and discoloration, significantly reducing the commercial quality and nutritional value of the corn (Prończuk et al., 1991; Opaterean et al., 2014). In the absence of effective resistant cultivars, the

management of maize stalk rot in maize predominantly depends on spraying chemical fungicides. Nevertheless, the extensive and prolonged application of systemic fungicides has resulted in the emergence of resistance (Fisher et al., 2018; Rancane et al., 2023). Therefore, the discovery of new fungicides that do not have cross-resistance with existing fungicides to control *Fusarium* disease in China is urgent.

Fludioxonil, a phenylpyrrole fungicide, has been confirmed to exhibit broad-spectrum activity against a wide range of fungal pathogens and has been used in field applications to control various diseases. It shows significant application value particularly in the management of maize diseases. For instance, it demonstrates effective inhibitory activity against pathogens such as *Pythium* spp. and *Fusarium* spp. (causative agents of maize stalk rot), as well as *F. graminearum* (the causal agent of maize ear rot). It is commonly applied via seed treatment or foliar spraying to reduce seedling rot, stalk rot, and grain mold caused by these diseases (Hai-Ming et al., 2014). At present, the mechanism of action of

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fludioxonil is not fully understood, and it is believed that it binds to Os1, a type III histidine kinase, and thus transmits signals to initiate the Hog1-MAPK cascade (Kojima et al., 2004; Alberoni et al., 2010; Duan et al., 2013). Previous studies have suggested that the mode of action of fludioxonil is to inhibit fungal growth by overstimulating the high osmolarity glycerol (HOG) stress response signal transduction pathway, which causes the hyphae to swell and burst (Corran et al., 2008). The HOG stress response pathway, a branched mitogen-activated protein kinase (MAPK) signal transduction system, has been well characterized in *Saccharomyces cerevisiae*, and is known to mediate osmoregulation in response to environmental factors (Hohmann, 2002; Stefan, 2009). A point mutation in the histidine kinase Os1 would not only confer resistance to fludioxonil in *Botrytis cinerea*, *Fusarium melongena*, and *Phomopsis longicolla*, but also, overexpression of the CORVET complex alleviates the fungicidal effects of fludioxonil on the yeast *S. cerevisiae* expressing hybrid histidine kinase 3 (Yoshimi et al., 2004; Furukawa et al., 2012; Randhawa et al., 2018; Brandhorst et al., 2019; Zhou et al., 2019; Zhou et al., 2020a; Wang et al., 2022; Ren et al., 2024).

Fludioxonil resistant mutants are more readily available under laboratory conditions (Han et al., 2017; Yin et al., 2024), whereas laboratory-obtained mutants usually show fitness costs, which may explain the difficulty in isolating resistant strains in the field. These laboratory mutants often involve point mutations in the type III histidine kinase Os1. However, aside from a few that have been confirmed, most of these mutations remain unverified (Wang et al., 2024; Wen et al., 2022; Shi et al., 2023). However, fludioxonil has been used for more than thirty years, but only few cases of resistance in the field have been reported to date (Jaafar and Sabine, 2016), despite the fact that resistant mutants could easily be obtained for many fungal species (*B. cinerea*, *Sclerotinia sclerotiorum*, *Aspergillus nidulans*, *Neurospora crassa*, *Ustilago maydis*) through continual exposure to high concentrations of fludioxonil (Vignutelli et al., 2002; Avenot et al., 2005; Taiwo et al., 2021). The fungicide resistance action committee (FRAC) categorizes fludioxonil as exhibiting low to moderate resistance (Kim et al., 2015). However, the resistance mechanism of fludioxonil has not yet been fully resolved.

In this study, gene knockout and overexpression techniques were employed to identify the *FgOs1* gene in *F. graminearum*. The results confirmed its involvement in regulating fludioxonil resistance. Furthermore, through the screening of resistant mutants, novel resistance loci on the *FgOs1* gene were uncovered, and the mycelial growth, conidiation, stress susceptibility, pathogenicity, and virulence of these resistant mutants were comprehensively analyzed. In this study, we explored the potential mechanism of resistance to fludioxonil in *F. graminearum*. This study advances the understanding of fludioxonil's mode of action in *F. graminearum* and provides a theoretical basis for developing integrated disease management strategies against corn stalk rot and ear rot, particularly in the context of emerging fungicide resistance.

## 2. Materials and methods

### 2.1. Medium, fungicides and fungal strain

PDA medium (200 g potato, 20 g dextrose, and 15 g agar per liter of distilled water) was used to analyze mycelia growth and sensitivity to stresses and cross-resistance (phenamacril, carbendazim, tebuconazole, fludioxonil and fluazinam). YEPD medium (3 g of yeast extract, 10 g of peptone, 20 g of D-glucose and 15 g of agar per liter of distilled water) was used for *F. graminearum* mycelium production. CMC medium (15 g sodium carboxymethyl cellulose, 1 g yeast extract, 0.825 g (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 1 g KH<sub>2</sub>PO<sub>4</sub>, 0.5 g MgSO<sub>4</sub>·7H<sub>2</sub>O per liter of distilled water) was used to measure conidiation.

Fludioxonil, phenamacril, tebuconazole, iprodione and procymidone were dissolved in dimethyl sulfoxide to obtain a stock solution of 10 mg of active ingredient/mL (10<sup>4</sup> µg/mL). Solution of carbendazim was prepared by dissolving the compound in hydrochloric acid. The stock

solutions were subsequently kept at 4 °C until required for use. The wild-type (WT) strain of *F. graminearum* was used for the construction of various gene deletion mutants.

### 2.2. Generation of fludioxonil-resistance mutants

Resistant mutants were induced by UV induction and pharmaceutical domestication (Fig. S2). Five clusters of WT strain extracted from 3 days of culture were inoculated into 50 mL flasks containing 30 mL of CMC medium and shaken for 5 days at 25 °C 160 rpm. Afterwards, the solution was filtered through a magic filter cloth into a 50 mL centrifuge tube and centrifuged at 5000 rpm for 10 min to obtain the conidial precipitate. Distilled water was added to the precipitate in the centrifuge tube and mixed thoroughly to obtain 1 × 10<sup>5</sup> conidia. 100 µL of 1 × 10<sup>5</sup> conidia were coated on PDA culture plates containing 10 µg/mL, 20 µg/mL and 30 µg/mL fludioxonil. Then the plates of each concentration of agent were irradiated with UV light for 0 s, 1 min and 5 min for induction. Strains were obtained after dark treatment of all plates at 28 °C for 7 days. Following UV induction, single colonies from fungicide-amended plates were subcultured three times on PDA with 10 µg/mL fludioxonil, discarding unstable or non-viable strains. Surviving strains were further subcultured twice on 20 µg/mL fludioxonil PDA to select those stably growing under higher fungicide pressure. Finally, these domesticated strains were validated for resistance stability on PDA with 10 µg/mL and 20 µg/mL fludioxonil, yielding heritably stable fludioxonil-resistant isolates that were preserved.

### 2.3. Determination of the mutation sites in *FgOs1*

The genomic DNA of fludioxonil-resistant mutant, replacement mutant,  $\Delta FgOs1$  and WT strain was extracted by CTAB (Duan et al., 2016), and primers were designed at 1500 bp upstream and downstream of the *FgOs1* gene to amplify the complete coding region of the *FgOs1* gene. The PCR products were analyzed by agarose gel electrophoresis and then sequenced by Tsingke Biotechnology Co., Ltd. Afterwards, the sequencing results were subjected to multiple sequence comparison.

### 2.4. Obtaining and transformation verification of resistance mutants

The double-joint PCR method was used to generate the gene replacement constructs (Yu et al., 2004). Briefly, the flanking regions at the 5' and 3' ends of each gene were amplified using primers listed in Table S1. These flanking sequences were fused with the hygromycin-resistance gene cassette (HPH) to generate overlapping PCR products. Using the polyethylene glycol (PEG)-mediated transformation method, DNA fragments were transferred into protoplasts of the WT *F. graminearum* strain (Tang et al., 2021a). Subsequently, the deletion mutants were further confirmed by PCR assay using relevant primers. For complementation, the corresponding open reading frame (ORF) with its native promoter was cloned into the GFP vector. The complementation construct *FgOs1*-GFP was subsequently introduced into the protoplasts of the  $\Delta FgOs1$  deletion mutant to generate the complementary strain  $\Delta FgOs1$ -C. Transformants were screened by geneticin (G418).

To construct overexpression mutants, the target gene was inserted into an overexpression vector pGTN with the same method. Protoplast transformation of *F. graminearum* as previously described, and all of the transformants selected using corresponding antibiotics were verified by PCR. Reverse vectors containing the Q140STOP or R1183STOP substitutions in *FgOs1* were constructed as described previously for complementation of *FgOs1* deletion mutants. Transformants were verified by PCR.

### 2.5. Phylogenetic and domain analyses

The amino acid sequences of Os1 were retrieved from the FungiDB (<https://fungidb.org/fungidb/app>). Subsequently, the domains of these

proteins were predicted using SMART (<http://smart.embl-heidelberg.de>) (Tang et al., 2021a). To construct a phylogenetic tree, we first obtained the homologous amino acid sequences of proteins of Os1 from multiple species from FungiDB, and then aligned them using ClustalW. Then, using MEGA 12 software and using the neighbor-joining method, we successfully constructed a phylogenetic tree.

## 2.6. Biological fitness of the mutants

### 2.6.1. Mycelial growth test

Mycelial growth of the WT and the resulting transformants was routinely maintained on potato dextrose agar medium (PDA), yeast extract peptone dextrose agar medium (YEPD), complete medium (CM) and minimal medium (MM) plates and the images were taken 3 days after inoculation.

### 2.6.2. Measurement of conidia production

For conidiation assay, 5 mycelial plugs (5 mm in diameter) of each isolate taken from the periphery of a 3-day-old colony were inoculated in a 50 mL flask containing 30 mL of CMC. Flasks were incubated at 25 °C for 5 days in a shaker (160 rpm). For each isolate, the number of conidia in the medium was determined using a hemacytometer. The experiment was repeated three times.

### 2.6.3. Sporulation and germination

As described earlier, 5 mycelial plugs from colonies were first placed in 50 mL flasks containing 30 mL of CMC and the conidia were obtained after 5 days of incubation in a shaker (160 rpm) at 25 °C.  $1 \times 10^6$  conidia were plated on separate plates containing different concentrations of the agents. The morphology of conidia was then observed and photographed at 2, 4 and 6 h of incubation.

### 2.6.4. Determination of germ tubes elongation inhibition

After 8 h, the colonies were observed under an inverted microscope, and the length ( $\mu\text{m}$ ) of the germ tubes of each group of colonies was measured and recorded with the measuring tape on the microscope, and the percentage of inhibition of germ tube growth was calculated. The experiment was repeated three times. Bud germ tubes elongation inhibition (%) = control germ tubes length - treated germ tubes length / control germ tubes length  $\times 100$  %.

## 2.7. Pathogenicity assays

In this study, to examine the virulence of each strain, we inoculated corn ears with fungal spore suspensions of each strain at a concentration of  $10^6$  conidia/mL (Huang et al., 2024). Firstly, the surface of the corn ears was carefully disinfected with 75 % ethanol. Subsequently, each corn kernel was individually wounded using a sterilized toothpick. Then, the fungal spore suspension was inoculated onto the corn kernels. After the inoculated corn ears were cultured in an incubator at 25 °C for 7 days, we took photos of the corn ears and analyzed their disease symptoms. Each strain in this experiment had three biological replicates.

Then, in the corn silk pathogenicity experiment, colonies of peripheral mycelial clumps (5 mm in diameter) were inoculated onto corn silk, which were then incubated in an incubator for 5 days before being photographed and analyzed for virulence (Tang et al., 2021b). Each strain in this experiment had 10 biological replicates.

For corn stalk infection assays, the corn was grown for 2 months and the corn stalk infection experiment was carried out (Jiang et al., 2025; Xia et al., 2024). Briefly, the concentration of conidial suspension of the strain was adjusted to  $10^6$  conidia/mL, and 10  $\mu\text{L}$  of the conidia suspension was injected into the middle of the third stalk of the above-ground stem of corn, and 0.05 % Tween-20 sterile water was used as a blank control. Symptoms were observed and photographed 14 days after inoculation.

## 2.8. Stress sensitivity assays

To analyze the sensitivity of various stresses, a 5 mm mycelial plug was taken from the colony edge of each strain and transferred onto PDA media amended with or without 0.2 g/L congo red (CR), 0.02 % sodium dodecyl sulfate (SDS), 0.7 M sodium chloride (NaCl), and 1 M potassium chloride (KCl), 1.5 M sorbitol, 0.5 M magnesium chloride ( $\text{MgCl}_2$ ), and 0.3 M calcium chloride ( $\text{CaCl}_2$ ), 0.01 M hydrogen peroxide ( $\text{H}_2\text{O}_2$ ). After incubated for 3 days at 25 °C, the colony diameter of each treatment with three replicates was measured. Each experiment was repeated three times.

## 2.9. Cross-resistance assay

These six *F. graminearum* strains were tested by inoculating them on PDA media with a triazole fungicide (tebuconazole), a cyanoacrylate-based fungicide (phenamacril), a benzimidazole fungicide (carbendazim) and the dicarboximide fungicide (iprodione and procymidone). The concentration of iprodione in the medium was 50  $\mu\text{g/mL}$ , the concentration of procymidone in the medium was 100  $\mu\text{g/mL}$ , and the concentration of other fungicides (tebuconazole, phenamacril, and carbendazim) in the medium was 5  $\mu\text{g/mL}$ . Meanwhile, the susceptibility of the fungicide to pyrrolic acid fungicide (fludioxonil) was determined by inhibiting the mycelial growth  $\text{EC}_{50}$  value of the WT strain and it was determined that the final concentration was 10  $\mu\text{g/mL}$ . There were three replicate plates for each treatment and the experiment was carried out three times.

## 2.10. Statistical analysis

Each experiment was repeated three times. Statistical analysis was performed using Data Processing System (DPS). Inter-group differences were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's honestly significant difference (HSD) test to control for multiple comparisons, with a significance threshold of  $P < 0.05$ . Means with different letters (e.g., "a" and "b") indicate significant differences, while means sharing the same letter are not significantly different.

## 3. Results

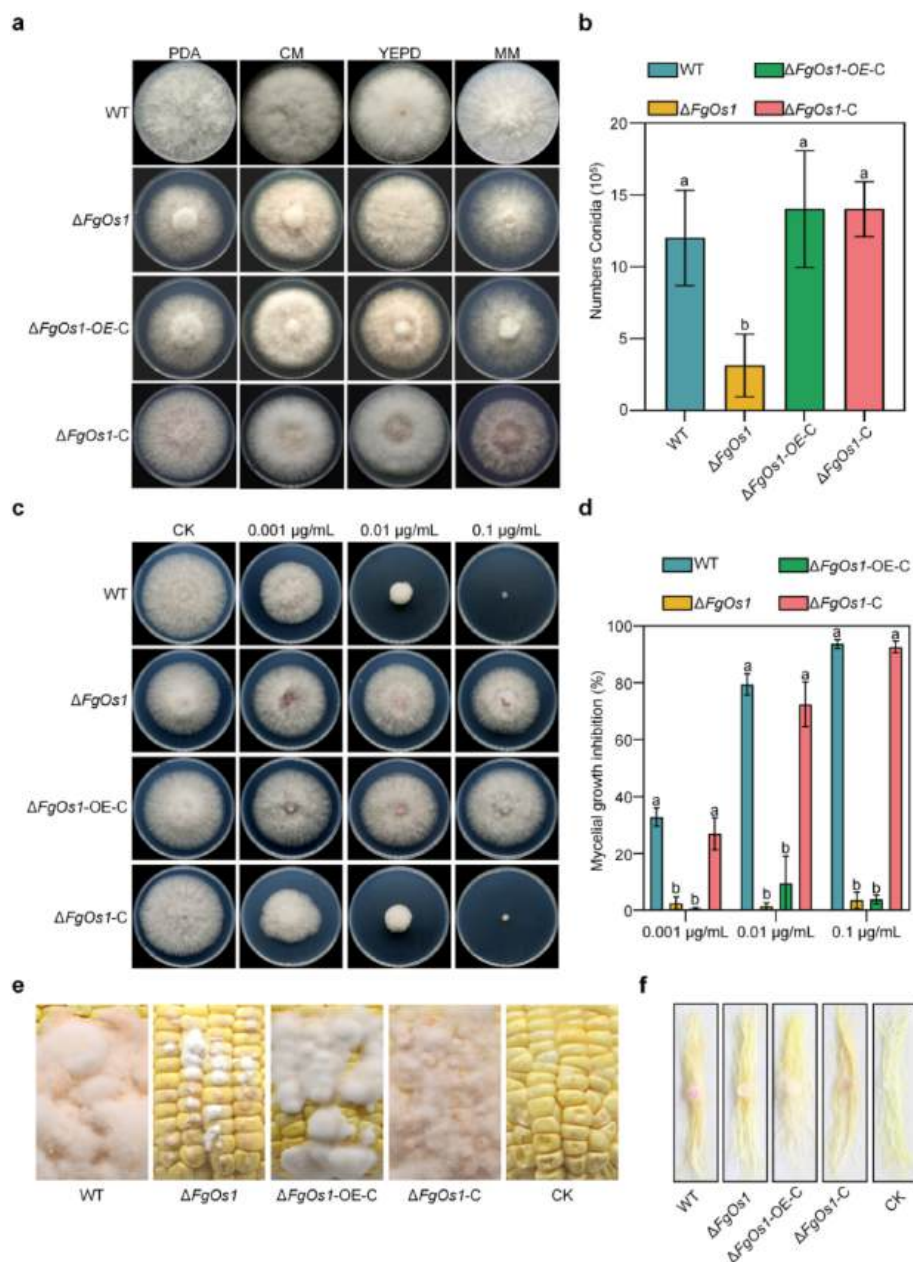
### 3.1. The antifungal activity of fludioxonil against *F. graminearum*

The antifungal activity of fludioxonil against *F. graminearum* was determined. The inhibitory effect of different concentrations of fludioxonil on the growth of *F. graminearum* mycelium was calculated (Fig. S1a). The  $\text{EC}_{50}$  (effective concentration for 50 % inhibition) and MIC (minimum inhibitory concentration) of fludioxonil against *F. graminearum* were 0.01  $\mu\text{g/mL}$  and 0.5  $\mu\text{g/mL}$ , respectively (Fig. S1b). In addition, to determine the effect of fludioxonil on conidial germination of *F. graminearum*, conidial germination morphology and shoot tube elongation were also evaluated after treatment with different concentrations of fludioxonil. Increasing concentrations of fludioxonil did not affect conidial morphology, but affected germ tube elongation, which was significantly reduced (Fig. S1c, d). Taken together, these results indicate that fludioxonil has strong antifungal activity against *F. graminearum* mycelial growth and conidial germination.

### 3.2. *FgOs1* is essential for hyphal growth, conidiation in *F. graminearum*

To assess the effect of *FgOs1* on the hyphal growth and conidia production of *F. graminearum*, we knocked out the *FgOs1* gene. The results showed that the growth rate of  $\Delta\text{FgOs1}$ , complementary strain  $\Delta\text{FgOs1-C}$  and overexpression strain  $\Delta\text{FgOs1-OE-C}$  showed similar colony morphology and growth rate to the WT after 3 days of growth on PDA, MM, CM and YEPD media at 25 °C (Fig. 1a). Conidia play an important role as propagules during the infection stage. Therefore, we





**Fig. 1.** Biological characterization of the fludioxonil-resistant mutant strain  $\Delta FgOs1$ . (a) The WT,  $\Delta FgOs1$ ,  $\Delta FgOs1$ -OE-C,  $\Delta FgOs1$ -C strains were grown on PDA, CM, YEPD and MM media at 25 °C for 3 days. (b) Conidiation of WT,  $\Delta FgOs1$ ,  $\Delta FgOs1$ -OE-C,  $\Delta FgOs1$ -C was measured. (c) Sensitivity testing of WT,  $\Delta FgOs1$ ,  $\Delta FgOs1$ -OE-C,  $\Delta FgOs1$ -C to fludioxonil. (d) Inhibition of mycelial growth of WT,  $\Delta FgOs1$ ,  $\Delta FgOs1$ -OE-C,  $\Delta FgOs1$ -C by various concentrations of fludioxonil. (e) Infected corn ears were assessed after 7 days of inoculation with conidial suspension of WT,  $\Delta FgOs1$ ,  $\Delta FgOs1$ -OE-C,  $\Delta FgOs1$ -C. (f) Infected corn silk were evaluated 5 days after inoculation with mycelial plugs of WT,  $\Delta FgOs1$ ,  $\Delta FgOs1$ -OE-C,  $\Delta FgOs1$ -C. Inter-group differences were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's honestly significant difference (HSD) test for multiple comparisons, with a significance level set at  $P < 0.05$ . Means marked with different letters (e.g., "a" and "b") indicate significant differences, while means sharing the same letter indicate no significant difference.

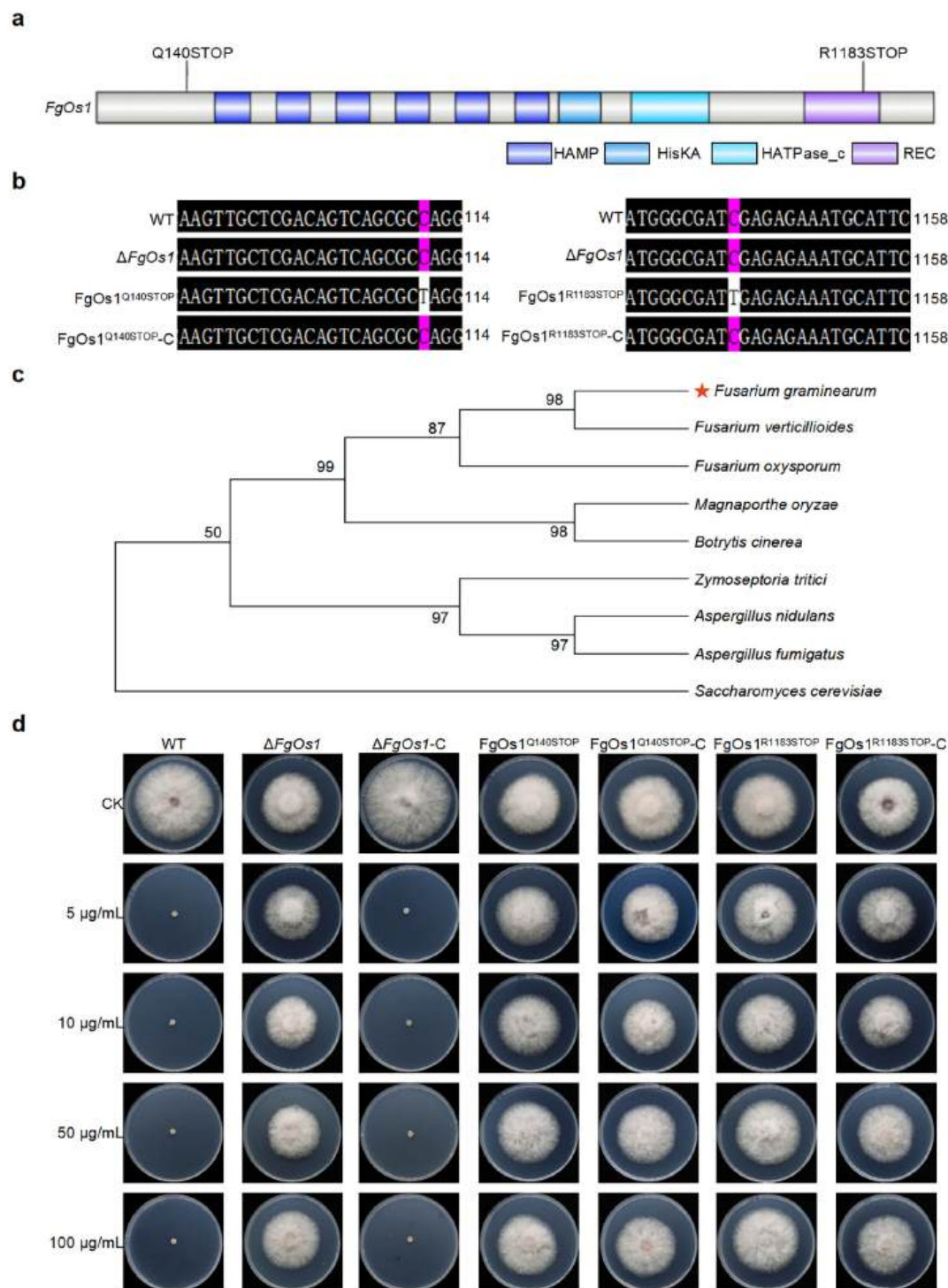
conducted a conidia production assay on *F. graminearum* to investigate the function of *FgOs1*. The results showed that  $\Delta FgOs1$  produced fewer conidia compared with the WT and  $\Delta FgOs1$ -C (Fig. 1b). And then to demonstrate the role of *FgOs1* in resistance of fludioxonil, the WT strain was used as a control, and the knockout mutant, inoculated with complementary strains ( $\Delta FgOs1$ -C and  $\Delta FgOs1$ -OE-C) on PDA media containing different concentrations of the fludioxonil agent, and it was found that the tolerance of  $\Delta FgOs1$  to fludioxonil stress was significantly enhanced, whereas interestingly, the overexpression of the expressing strain  $\Delta FgOs1$ -OE-C was also resistant to fludioxonil (Fig. 1c, d). To investigate the effect of fludioxonil resistant mutants on the virulence of *F. graminearum*, 7 days after inoculation with *F. graminearum* conidial

suspensions, it was observed that the *FgOs1* deletion mutant caused significantly less disease symptoms on corn cobs than the WT strain, the replacement strain  $\Delta FgOs1$ -C, and the overexpression strain  $\Delta FgOs1$ -OE-C, and greater than the untreated CK strain, and the overexpression strain was also slightly smaller than the WT strain (Fig. 1e). Similarly, 5 days after inoculation with *F. graminearum* plugs, the area of lesion caused by  $\Delta FgOs1$  was also significantly smaller than that caused by WT strain on corn whisks (Fig. 1f). These results suggest that *FgOs1* is essential for conidia production, fludioxonil resistant and virulence in *F. graminearum*.

### 3.3. Generation and characterization of fludioxonil-resistant mutants

Two fludioxonil-resistant mutants were generated via UV mutagenesis and fungicide acclimation in *F. graminearum*. Sequence analysis of *FgOs1* revealed two nonsense mutations in separate mutant strains: A premature termination codon (PTC) at glutamine-140 (Q140) caused by a CAG → TAG transition at nucleotide position 418, designated as

*FgOs1*<sup>Q140STOP</sup> which truncates the protein upstream of the conserved kinase domain. A PTC at arginine-1183 (R1183) resulting from a CGA → TGA transversion at nucleotide 3547, designated as *FgOs1*<sup>R1183STOP</sup>, which disrupts the REC receiver domain essential for phosphorelay signaling (Fig. 2a,b).



**Fig. 2.** Identification of resistance mutants. (a) A schematic representation of the domains of *FgOs1*, which includes the HAMP (histidine kinase, adenylyl cyclases, methyl binding proteins, and phosphatases) domain, the HisKA (phosphoacceptor) domain, the HATPase\_c (histidine kinase-like ATPases) domain, and the REC (receiver domain). (b) Comparison of *FgOs1* amino acid mutations in a partial fragment between fludioxonil-resistant strains and fludioxonil-sensitive strains. (c) Phylogenetic analyses of the amino acid sequences of *FgOs1* from *F. graminearum*, and a comparison of the amino acids identical to those in other fungal homologous genes. (d) Sensitivity of the WT,  $\Delta FgOs1$ , *FgOs1*<sup>Q140STOP</sup> and *FgOs1*<sup>R1183STOP</sup> mutants to fludioxonil. PDA media were incubated at 25 °C for 3 days.

### 3.4. Frequency and conservation analysis of *FgOs1* mutations

Following UV mutagenesis and fungicide acclimation of the WT strain, two fludioxonil-resistant mutants (*FgOs1*<sup>Q140STOP</sup> and *FgOs1*<sup>R1183STOP</sup>) were isolated from *F. graminearum* with a mutation frequency of 0.004 %. Genomic sequencing confirmed that both mutants harbored nonsense mutations in the *FgOs1* histidine kinase gene. Sequence alignment and phylogenetic analysis revealed that the mutated residues (Q140 and R1183) are evolutionarily conserved across diverse fungal pathogens, including *Fusarium verticillioides*, *Fusarium oxysporum*, *Magnaporthe oryzae*, *Botrytis cinerea*, *Zymoseptoria tritici*, *Aspergillus nidulans*, *Aspergillus fumigatus*, and *Saccharomyces cerevisiae* (Fig. 2c). After ten consecutive transfers on fungicide-free PDA medium, all fludioxonil-resistant mutants retained their ability to grow on PDA medium amended with 10 µg/mL fludioxonil, indicating stable resistance. For the two *F. graminearum* mutants (*FgOs1*<sup>Q140STOP</sup> and *FgOs1*<sup>R1183STOP</sup>), the median effective concentration (EC<sub>50</sub>) values remained >100 µg/mL in both the 1st and 10th generations, with resistance factors (RF) exceeding 1000-fold compared to the WT strain (Table 1). These results confirm their high-resistance (HR) phenotype to fludioxonil.

### 3.5. Mutations in *FgOs1* confer fludioxonil resistance in *F. graminearum*

To elucidate the functional role of *FgOs1* gene mutations in fludioxonil resistance, a series of genetic complementation experiments were conducted. Using a homologous double-exchange strategy, replacement mutants of *FgOs1* with the Q140STOP and R1183STOP mutations (designated as *FgOs1*<sup>Q140STOP</sup>-C and *FgOs1*<sup>R1183STOP</sup>-C) and their WT counterparts were successfully constructed. These mutants were verified by PCR amplification (Fig. 2b). Subsequently, the sensitivity of the WT strain, the original resistant mutants ( $\Delta FgOs1$ , *FgOs1*<sup>Q140STOP</sup> and *FgOs1*<sup>R1183STOP</sup>), and replacement mutants (*FgOs1*<sup>Q140STOP</sup>-C and *FgOs1*<sup>R1183STOP</sup>-C) to 10 µg/mL fludioxonil (Fld) were systematically evaluated to determine their resistance levels. Notably, the WT strain and the complemented control ( $\Delta FgOs1$ -C) failed to grow on the medium supplemented with 10 µg/mL fludioxonil. In contrast, the  $\Delta FgOs1$  mutant, the *FgOs1*<sup>Q140STOP</sup> and *FgOs1*<sup>R1183STOP</sup> mutants, along with their corresponding replacement mutants, exhibited normal growth at this concentration (Fig. 2d). Collectively, these findings strongly indicate that the Q140STOP or R1183STOP mutations within the *FgOs1* gene are responsible for conferring fludioxonil resistance to *F. graminearum*.

### 3.6. Biological fitness analysis of fludioxonil-resistant mutants

Biological fitness parameters, such as mycelial growth, conidia production, and pathogenicity, were comprehensively analyzed in fludioxonil-resistant mutants. In terms of mycelial growth, after

incubation on PDA medium at 25 °C for 3 days, the mycelial growth rates of the two resistant mutants showed no significant difference from the WT strain (Fig. 2d). Regarding conidium production, the conidial morphology of the resistant mutants was indistinguishable from that of the WT strain. However, the conidiation levels of both the resistant mutants and the replacement mutants were significantly lower compared to the WT strain (Fig. 3a). In terms of pathogenicity, seven days after inoculation, all the resistant mutants induced smaller disease symptoms on the inoculated corn ear compared to the WT strain (Fig. 3b). Similarly, on corn silk, the lesion sizes caused by the resistant mutants were significantly smaller than those caused by the WT strain (Fig. 3c).

### 3.7. Sensitivity to various stresses

The sensitivity of the resistant strains *FgOs1*<sup>Q140STOP</sup>, *FgOs1*<sup>R1183STOP</sup>, the alternative strains *FgOs1*<sup>Q140STOP</sup>-C, *FgOs1*<sup>R1183STOP</sup>-C, and the WT strain to osmotic stress, metal ion stress, oxidative stress, and cell wall stress was evaluated. The results showed that in vitro application of osmotic stress factors resulted in a significant decrease in the growth rate of the resistant mutants, virtually no colony expansion and significant inhibition of aerial mycelium production compared to WT and  $\Delta FgOs1$ -C. PDA medium supplemented with 0.7 M NaCl, 1 M KCl, and 1.5 M Sorbitol resulted in less than 40 % inhibition of both the WT and  $\Delta FgOs1$ -C strains, whereas inhibition of the resistant mutant and the substitution mutant could be as high as 80 % or more. The presence of the point mutation resulted in an up-regulation of the osmotic stress factor for *F. graminearum* growth inhibition to as much as 2-fold (Fig. 4a, b). In tolerance to metal ion stress, it was found that the growth rate of the resistant mutant and the replacement mutant on PDA medium supplemented with 0.5 M CaCl<sub>2</sub> and 0.5 M MgCl<sub>2</sub> was significantly reduced and the expansion of colonies was severely inhibited compared to WT and  $\Delta FgOs1$ -C (Fig. 4a, b). In contrast, colony growth on PDA medium supplemented with 10 mM H<sub>2</sub>O<sub>2</sub>, 0.01 % SDS, and 10 g/L CR was not significantly inhibited, suggesting that the point mutation is not involved in regulating the response of *F. graminearum* to oxidative stress and cell wall stress (Fig. S3a, b). This phenotypic analysis underscores the critical role of *FgOs1* in mediating osmotic and ionic homeostasis in *F. graminearum*, while implicating separate genetic pathways in oxidative and cell wall stress responses.

### 3.8. Cross-resistance of fludioxonil-resistant mutants

Cross-resistance profiles of *FgOs1*<sup>Q140STOP</sup>-C and *FgOs1*<sup>R1183STOP</sup>-C mutants were rigorously assessed against fungicides representing four distinct modes of action: the triazole tebuconazole (ergosterol biosynthesis inhibitor), the phenamacril (myosin cytoskeleton disruptor), the benzimidazole carbendazim (β-tubulin polymerization inhibitor), and iprodione, procymidone (histidine kinase inhibitor). Despite exhibiting complete resistance to 10 µg/mL fludioxonil (Fig. 5), both mutants displayed WT-level sensitivity to all tested fungicides. Meanwhile, we also observed that the resistant mutants exhibit the same level of resistance to the dicarboximide fungicides procymidone and iprodione at higher concentrations as they do to fludioxonil. This is not surprising because the dicarboximides are known to share similar modes of action with fludioxonil and also target the HOG-MAPK kinase signal transduction pathway. In contrast, the WT and complemented  $\Delta FgOs1$ -C strain were fully inhibited by other fungicide at field-relevant concentrations, confirming restoration of susceptibility upon genetic complementation.

## 4. Discussion

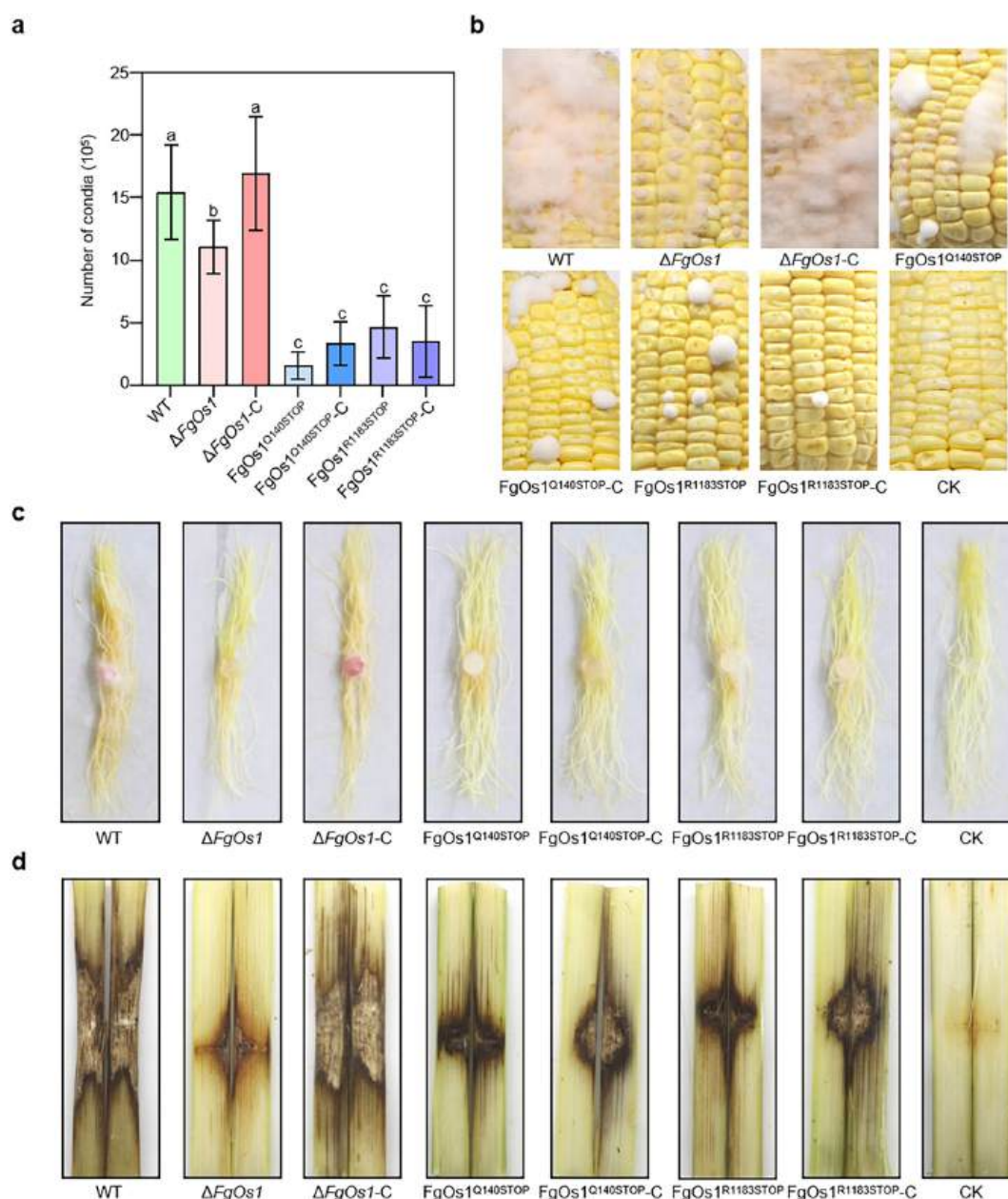
Given the increasingly serious problem of fungicide resistance in the field, it is urgent to introduce fungicides with novel modes of action to control resistant populations. Fludioxonil, a signaling disruptor, has

**Table 1**  
Genetic stability of fludioxonil by point mutant strains and their replacement strains.

Strains	Phenotype <sup>a</sup>	First generation		Tenth generation	
		EC <sub>50</sub> (µg/mL)	RF	EC <sub>50</sub> (µg/mL)	RF
WT	S	0.01	–	0.01	–
$\Delta FgOs1$	R	>100	>1000	>100	>1000
$\Delta FgOs1$ -C	S	0.01	–	0.01	–
<i>FgOs1</i> <sup>Q140STOP</sup>	R	>100	>1000	>100	>1000
<i>FgOs1</i> <sup>Q140STOP</sup> -C	R	>100	>1000	>100	>1000
<i>FgOs1</i> <sup>R1183STOP</sup>	R	>100	>1000	>100	>1000
<i>FgOs1</i> <sup>R1183STOP</sup> -C	R	>100	>1000	>100	>1000

<sup>a</sup> “S” indicates sensitive strains; “R” indicates resistant strains; “–” represents no data.

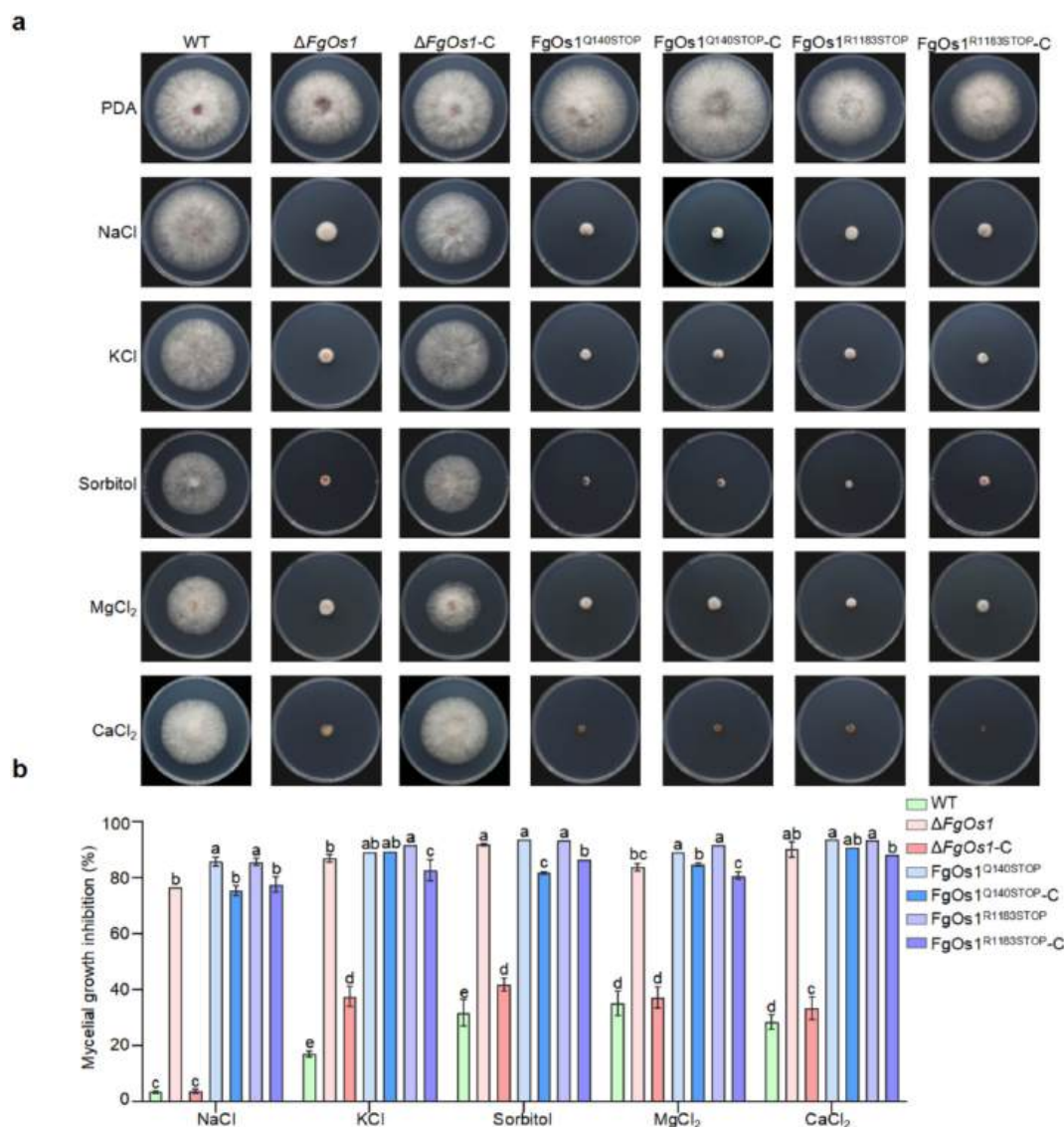




**Fig. 3.** Fitness of fludioxonil-resistant *F. graminearum* Mutants. (a) Conidiation of WT,  $\Delta FgOs1$ ,  $\Delta FgOs1$ -C,  $FgOs1^{Q140STOP}$ ,  $FgOs1^{Q140STOP}$ -C,  $FgOs1^{R1183STOP}$  and  $\Delta FgOs1^{R1183STOP}$  was measured. (b) The deletion mutants of  $\Delta FgOs1$ ,  $FgOs1^{Q140STOP}$  and  $FgOs1^{R1183STOP}$  showed significantly reduced virulence on corn ears. Infected corn ears were assessed after 7 days of inoculation with conidial suspension of WT,  $\Delta FgOs1$ ,  $FgOs1^{Q140STOP}$ ,  $FgOs1^{R1183STOP}$  and complemented strains. (c) Fludioxonil-resistant strains were tested for virulence to corn silk. Infected corn silk were evaluated 5 days after inoculation with mycelial plugs of WT,  $\Delta FgOs1$ ,  $FgOs1^{Q140STOP}$ ,  $FgOs1^{R1183STOP}$  and complemented strains. (d) Fludioxonil-resistant strains were tested for virulence to corn stems. Infected corn stems were assessed after 14 days of inoculation with conidial suspension of WT,  $\Delta FgOs1$ ,  $FgOs1^{Q140STOP}$ ,  $FgOs1^{R1183STOP}$  and complemented strains.

been used worldwide for the control of numerous crop diseases, including rice blast, apple canker, and rape sclerotinia rot (Motoyama et al., 2008; Zhao et al., 2010; Kuang et al., 2011; Qiu et al., 2018). The mechanism of action of fludioxonil is reported to be involved in regulating the high osmolarity glycerol (Hog1) cascade of mitogen-activated protein kinase (MAPK) signaling pathway via activating both transport-associated proteins phosphorylation and glycerol synthesis, and ultimately disturbing signal transmission (Wen et al., 2022). The MAPK cascades contain MAPK kinase kinases (MAPKKKs), MAPK kinases (MAPKKs), and MAPKs, which include an N-terminal regulatory domain and a C-terminal serine/threonine protein kinase domain. The modifications leading to fludioxonil-resistance (Flu<sup>R</sup>) not only concentrate in the HAMP domain of group III HK Os1, but also on other key proteins of

the HOG pathway including Os4, Os5 and Os2 (Dry et al., 2004; Avenot et al., 2005; Ochiai et al., 2010; Duan et al., 2014; Ren et al., 2016; Chen et al., 2020). In this study, two fludioxonil-resistant mutants were obtained by UV-induced conidial mutagenesis, and sequencing results showed that the mutants were mutated in the Os1 gene, with one of the point mutations,  $FgOs1^{Q140STOP}$ , not being in any of the structural domains, and the point mutation,  $FgOs1^{R1183STOP}$ , being in the REC structural domain (Fig. 2a). Resistance was subsequently verified to be associated with the point mutations by genetic transformation and overexpression (Fig. 2d). Moreover, our data also indicate that no alterations occurred in other key proteins of the HOG pathway in these two resistant mutants. In contrast to the previous results of resistance caused by point mutations in the Os1 gene, our results show that



**Fig. 4.** Sensitivity to various stresses. (a) Mycelial growth status of WT,  $\Delta FgOs1$ ,  $\Delta FgOs1-C$ ,  $FgOs1^{Q140STOP}$ ,  $FgOs1^{Q140STOP-C}$ ,  $FgOs1^{R1183STOP}$  and  $FgOs1^{R1183STOP-C}$  strains under osmotic stress and metal ions stress. (b) Statistics of inhibition rate after osmotic stress and metal ions stress.

stopping translation of the *Os1* gene can also cause resistance to fludioxonil. The *FgOs1* knockout mutant showed sensitivity to fludioxonil, further clarifying that the inhibitory activity of fludioxonil is heavily dependent on the HOG1-MAPK pathway.

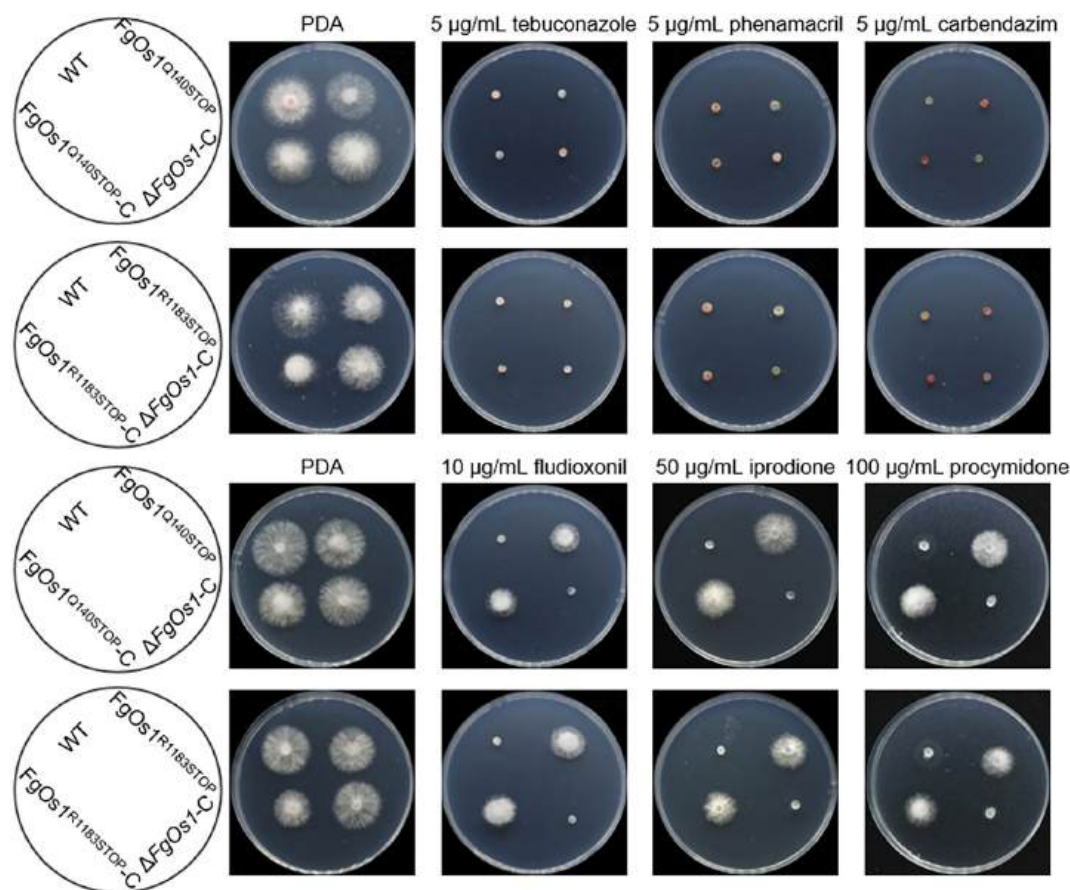
Previous fungicide resistance studies have shown that the development of resistance to fungicides by phytopathogenic fungi is accompanied by an adaptation cost. In this study, an adaptation penalty was also found in the fludioxonil-resistant mutants. We found that *FgOs1* knockout mutants, along with stop-translating mutants, were lower than the parents in terms of spore production and virulence (Fig. 1b, e, f) (Fig. 3). Interestingly, strains overexpressing *FgOs1* would remain resistant to fludioxonil in a state of restored spore and virulence production capacity (Fig. 1c, d). A critical finding of this study is that both knockout and overexpression of *FgOs1* confer comparable fludioxonil resistance in *F. graminearum*, a phenomenon likely arising from perturbed osmotic stress signaling homeostasis mediated by *FgOs1*. As a core regulator of osmotic stress pathways—whose hyperactivation underpins fludioxonil's toxicity—*FgOs1* requires precise expression to maintain signaling equilibrium: knockout ablates downstream transduction entirely, while overexpression saturates or desensitizes key components, with both scenarios impairing pathway regulation and

precluding toxic hyperactivation (De Ramon-Carbonell and Paloma, 2020; Zhou et al., 2024). In conclusion, resistance induced by *FgOs1* point mutations is often accompanied by fitness penalties, which puts the pathogen at a competitive disadvantage in the field, and this is the reason why little field resistance has been reported over the nearly 30 years of fludioxonil use.

The Hog1 pathway plays a key role in *F. graminearum* conidia formation and adversity stress, and the *Os1* termination translation resistance mutants had similar fitness for survival as the *Os1* knockout mutants, and were both more sensitive to osmotic stress than the sensitive strain (Fig. 4a, b), which is also in line with the results of previous studies (Ren et al., 2016; Qiu et al., 2018; Brandhorst et al., 2019; Zhou et al., 2020b). Similarly, all the fludioxonil-resistant strains were also involved in mediating metal ion stress, but not in regulating cell wall stress and oxidative stress (Fig. S3).

Even though it has been widely reported in the literature that fludioxonil-resistant mutants have low biological fitness and are difficult to survive in the field. However, with the impact of climate change and changes in farming systems, the presence of *FgOs1* point mutant strains is still detected in the field, and in this study, the fludioxonil-resistant mutants still have a certain degree of pathogenicity, which implies





**Fig. 5.** Cross-resistance between fludioxonil and other fungicides. Mycelial growth of fludioxonil-resistant strains on PDA media containing different concentrations of fludioxonil, tebuconazole, phenamacril, carbendazim, iprodione and procymidone. The concentration of fludioxonil in the medium was 10 µg/mL, the concentration of iprodione in the medium was 50 µg/mL, the concentration of procymidone in the medium was 100 µg/mL, and the concentration of other fungicides (tebuconazole, phenamacril, and carbendazim) in the medium was 5 µg/mL.

that the fludioxonil-resistant mutants still have the potential risk of forming a resistant population in the field. Therefore, in this study, the susceptibility of fludioxonil-resistant mutants to fungicides with other mechanisms of action was determined. In this study, cross-resistance analysis showed that laboratory-induced resistant mutants had no cross-resistance to fungicides with different modes of action, including carbendazim, tebuconazole and phenamacril, implying that the above fungicides can be used for the management of resistant populations (Fig. 5). Observation reinforces findings from other pathogens, where fludioxonil-resistant strains demonstrate cross-resistance exclusively to fungicides within the same signaling-blocking category (Malandrakis et al., 2013; Chen et al., 2022). During production, fludioxonil can be mixed or rotated with the above fungicides to delay the development of resistant populations.

In this study, we found that point mutations of Q140STOP and R1183STOP on *FgOs1* were associated with fludioxonil resistance, and the mutants were less biologically fit than the wild-type strain. However, there is still a risk of forming a resistant population in the field, which can be managed by mixing or rotating with fungicides with different mechanisms of action, such as tebuconazole, phenamacril, and carbendazim, for fludioxonil resistance. The results of this study deepen the understanding of fludioxonil and provide theoretical basis and data support for the development of integrated management strategies for corn ear rot.

#### CRedit authorship contribution statement

**Yaru He:** Writing – original draft, Investigation, Data curation.

**Zikuo Li:** Investigation, Data curation. **Ziyan He:** Investigation. **Aoran Wang:** Investigation. **Xuheng Gao:** Writing – original draft. **Haoxue Xia:** Writing – original draft, Supervision. **Shanyue Zhou:** Supervision. **Wende Liu:** Resources, Funding acquisition, Conceptualization. **Guangfei Tang:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization.

#### Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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#### Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.pestbp.2025.106633>.

## Data availability

The datasets and materials supporting the results are included within the article, and the work described has not been published before.

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