Phytopathology Research

RESEARCH

Open Access

Functional analysis of all succinate dehydrogenase subunits in *Fusarium fujikuroi*

Mengyuan Fan[1], Hao Qi[1], Wenyong Shao[1], Hao Zhang[2], Yanni Yin[1], Yun Chen[1], Youfu Zhao[3] and Zhonghua Ma[1*]

Abstract

Fusarium fujikuroi, the causal agent of rice bakanae disease (RBD), contains five succinate dehydrogenase (Sdh) subunits: FfSdhA, FfSdhB, FfSdhC1, FfSdhC2, and FfSdhD. However, the role of these subunits in regulating sensitivity to succinate dehydrogenase inhibitors (SDHIs) is largely unknown. Here, we conducted targeted gene disruption and phenotypic assays for all Sdh subunits and found that the deletion mutants of *FfSdhA*, *FfSdhB*, and *FfSdhD* exhibited severe defects in hyphal growth, conidiation, virulence, and sensitivity to $CaCl_2$ and oxidative stresses. To a lesser extent, the mycelial growth rate and conidial production of Δ*FfSdhC1* were also decreased as compared to those of the wild-type strain JS16. In addition, fungicide sensitivity assays showed that deletion of *FfSdhA*, *B*, *C1*, or *D* led to decreased sensitivity to all SDHIs tested. Unexpectedly, we were unable to obtain a *FfSdhC1* + *C2* double mutant and further found significant up-regulation of *FfSdhC2* in Δ*FfSdhC1*, indicating that *FfSdhC1* and *-C2* might be essential for fungal growth although the *FfSdhC2* deletion mutant was indistinguishable from the wild-type strain. These findings provide useful information for enhancing our understanding of the biological functions of the Sdh subunits in pathogenic fungi.

Keywords *Fusarium fujikuroi*, Rice bakanae disease, Succinate dehydrogenase inhibitors (SDHIs), Succinate dehydrogenase (Sdh) subunit

Background

Rice bakanae disease (RBD), caused mainly by *Fusarium fujikuroi*, is a devastating disease of rice worldwide (Asmaul et al. 2020). This pathogenic fungus produces secondary metabolites, including gibberellin A3, fusaric acid, and fumonisins, which pose a threat to human and animal health (Amatulli et al. 2010; Fiyaz et al. 2016). Over the past few decades, changes in climate and cropping systems have exacerbated RBD epidemics in many rice-growing regions (Bashyal et al. 2016). Unfortunately, no RBD-resistant cultivars are available, and controlling the disease relies heavily on seed treatment with fungicides. However, the extensive use of systemic fungicides has led to a significant increase in fungicide resistance in *F. fujikuroi*. For instance, the benzimidazole fungicide carbendazim, which was once effective in controlling RBD, has lost its efficacy in China (Chen et al. 2014). Since then, the imidazole fungicide prochloraz has been used extensively for disinfection of *F. fujikuroi* in rice seeds (Kim et al. 2010), but prochloraz-resistant isolates have also frequently been detected in many regions (Leroux and Gredt 1997; Yin et al. 2009). Recently, a new myosin inhibitor fungicide called phenamacril has been registered for the control of RBD and has shown excellent

*Correspondence:
Zhonghua Ma
zhma@zju.edu.cn
[1] State Key Laboratory of Rice Biology, Key Laboratory of Molecular Biology of Crop Pathogens and Insects, Institute of Biotechnology, Zhejiang University, Hangzhou 310058, China
[2] The Institute of Plant Protection, Chinese Academy of Agricultural Sciences, Beijing 100193, China
[3] Department of Plant Pathology, Irrigated Agriculture Research and Extension Center, Washington State University, Prosser, WA 99350, USA

efficacy (Zhang et al. 2015). Unexpectedly, resistant *F. fujikuroi* isolates have also been detected in the field of Zhejiang Province of China since 2016 (Hou et al. 2018). Therefore, there is an urgent need to introduce new fungicides to control RBD in China and other countries around the world.

The new generation of SDHIs can efficiently inhibit the energy metabolism of many pathogenic ascomycetes and basidiomycetes. Previous studies have demonstrated that several SDHIs, including pydiflumetofen and cyclobutrifluram, are highly active against *Fusarium* spp. (Shao et al. 2022; Liu et al. 2023; Sun et al. 2024). SDHIs act on the mitochondrial respiratory electron transport chain complex II, succinate dehydrogenase, which is composed of four subunits SdhA, -B, -C, and -D (Sdh1–4 in yeast) in eukaryotes. This process contributes to the proton gradient by supplying reducing equivalents resulting from succinate metabolism, and the reducing equivalents are then transported through the ubiquinone pool (Moosavi et al. 2019; Wu et al. 2023). To date, several studies have shown that mutations in subunits of SdhA, SdhB, SdhC, and SdhD may confer SDHI resistance in *Fusarium* and other various phytopathogenic fungi. In *Fusarium pseudograminearum*, FpSdhC1-A83V or FpSdhC1-R86K point mutation confers pydiflumetofen resistance (Li et al. 2023). Mutations of FaSdhB-H248Y, FaSdhC1-A64V, and FaSdhC1-R67K lead to resistance of *Fusarium asiaticum* to pydiflumetofen (Chen et al. 2021). Sun and colleagues found that some *F. fujikuroi* strains showed natural resistance to penflufen, which is mainly caused by amino-acid substitutions (Q46K, R283H, G430A, and Q586R) in SdhA (Sun et al. 2021). In addition, point mutations, including SdhB-H248L/D/Y, SdhC2-A83V/H144Y, SdhC2-G80R/A83V, and SdhD-S106F/E166K, are responsible for SDHI resistance in *F. fujikuroi* (Liu et al. 2023; Xue et al. 2023). However, the biological functions of the Sdh subunits have not yet been systematically documented in a phytopathogenic fungus.

To understand the biological function of all Sdh subunits in *Fusarium* spp., we carried out a gene deletion analysis for each of the five Sdh subunits in *F. fujikuroi*. Our results showed that *FfSdhA*, *B*, *C1*, and *D* deletion mutants exhibited resistance to all SDHIs tested, including pydiflumetofen, penflufen, and fluopyram. Moreover, *FfSdhA*, *B*, and *D*, but not *FfSdhC1* and *-C2*, played critical roles in regulating hyphal growth, conidiation, virulence, and sensitivity to $CaCl_2$ and oxidative stresses. In addition, *FfSdhC1* also showed some defects in conidiation and sensitivity to SDHIs. Although *FfSdhC2* deletion did not show detectable phenotypic changes compared to the wild-type strain, we failed to obtain a dual mutant of *FfSdhC1+C2.* In addition, the expression of *FfSdhC2* increased dramatically in the SdhC1 deletion mutant compared to the wild-type. These results indicate that two paralogous *FfSdhC* genes are partially functional complementary in *F. fujikuroi*.

Results

The FfSdh subunits in *F. fujikuroi*

To determine whether the putative *Sdh* genes are expressed properly in *F. fujikuroi*, we first downloaded the *SdhA* (accession No. FFUJ_02582), *SdhB* (FFUJ_08745), *SdhC1* (FFUJ_08951), *SdhC2* (FFUJ_12139), and *SdhD* (FFUJ_00494) nucleotide sequences from Fungi DB (https://fungidb.org/fungidb/app), and identified the positions of introns and protein domains for these Sdh subunits. The nucleotide sequences of *FfSdhA*, *-B*, *C1*, *-C2*, and *-D* genes are 2272, 960, 660, 687, and 679 bp in length, respectively. The *FfSdhA* gene contains five introns and encodes 649 amino acids. For *FfSdhB, FfSdhC1, FfSdhC2*, and *FfSdhD*, each bears two introns (Additional file 1: Figure S1a), and encodes 278, 187, 192, and 182 amino acids, respectively (Additional file 1: Figure S1b). The locations of the introns in the *FfSdh* genes were verified by sequencing the 1802, 247, 180, 215, and 365-bp cDNA. Reverse transcription PCR results showed that *FfSdhA, B, C1, C2* and *D* genes were able to be transcribed in *F. fujikuroi* (Fig. 1).

F. fujikuroi and other *Fusarium* spp. contains paralogous SdhC1 and -C2

Alignment assay showed that the deduced amino acids of FfSdhC1 exhibit a low degree of sequence identity (36.98%) with those of FfSdhC2 (Additional file 1: Figure S2a). Both FfSdhC1 and FfSdhC2 have three predicted transmembrane segments, specifically at the residues 83–106, 131–53, and 164–84 for FfSdhC1 and at the

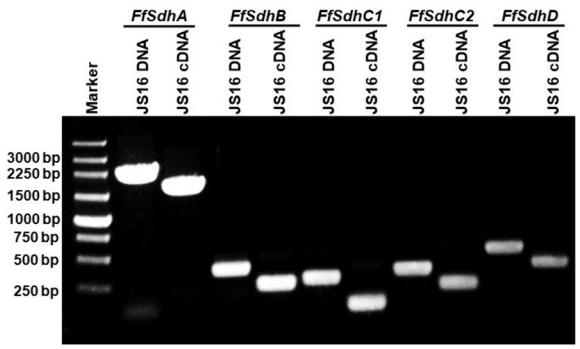

Fig. 1 Identification of introns and expression of *SDHs* in *F. fujikuroi*. Amplification of *FfSdhA*, *B*, *C1*, *C2*, and *D* gene from genomic DNA and cDNA of the wild-type isolate JS16 using primers A-F + A-R, B-F + B-R, C1-F + C1-R, C2-F + C2-R, and D-F + D-R. The primer pairs generated the expected 2124, 370, 276, 323 and 495 bp from genomic DNA and 1,802, 247, 180, 215, and 365 bp from cDNA, respectively

residues 82–109, 135–159, and 173–188 for FfSdhC2. They were predicted to localize in mitochondria by using the WoLF PSORT. Moreover, several other *Fusarium* species also contain two paralogous SdhCs with high identities for FfSdhC1 and -C2 (Additional file 1: Figure S2b, c), but the number of SdhC orthologues showed variation (1 to 3) among non *Fusarium* fungi (Additional file 1: Figure S2d, e). These results indicate that the orthologs of SdhC1 and SdhC2 are conserved in *Fusarium* spp.

Characterization of the FfSdh subunit mutants and expression of the *SdhC* genes

To investigate the functions of each FfSdh subunit in *F. fujikuroi*, we generated the gene deletion mutants using a homology recombination strategy (Additional file 1: Figure S3a, b). For each *Sdh* gene, at least two deletion mutants with similar phenotypes were identified by PCR with the corresponding primer pairs (Additional file 2: Table S1). As shown in Fig. 2a, the *FfSdhA*, *B*, and *D* deletion mutants grew dramatically slower than the wild-type JS16. The growth defect of Δ*FfSdhA*, *-B*, *-D* was restored to a wild-type level in the complementation strain, indicating that the hyphal growth defect was due to the deletion of *SdhA*, *B*, *D* gene. The *FfSdhC1* deletion mutant had only a 17.93% reduction in hyphal growth compared to JS16 on PDA plates (Fig. 2b), while no growth defect was observed for the *FfSdhC2* deletion mutant. These results indicate that *FfSdhA*, *B*, and *D*, and to a lesser extent, *FfSdhC1*, play an important role in *F. fujikuroi* growth.

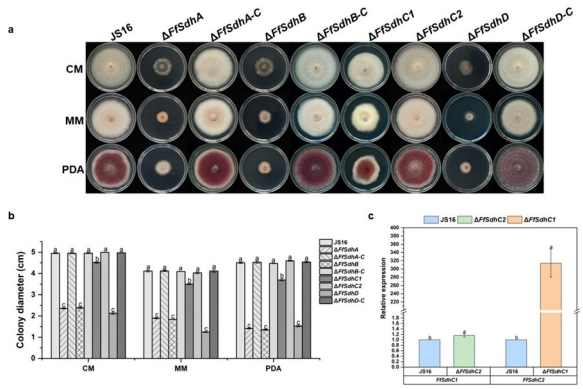

Fig. 2 Impact of the *FfSdh* deletion mutants on colony morphology and relative expression of *FfSdhC1* and *FfSdhC2* in the *FfSdhC* deletion mutants. **a** The wild-type JS16, *FfSdhA*, *B*, *C1*, *C2*, and *D* deletion mutants and complementation strains Δ*FfSdhA-C*, Δ*FfSdhB-C*, and Δ*FfSdhD-C* were grown on CM, MM, PDA at 25℃ for 7 days. **b** Mycelial growth diameter of each strain on CM, MM, and PDA plate was determined after 7 days of incubation. Line bars in each column denote the standard errors of three repeated experiments. Different letters represent statistically significant differences for each medium at $P = 0.05$ according to the one-way ANOVA test. **c** Relative expression of *SdhC* genes in each mutant is the relative amount of mRNA of each gene in the wild-type JS16. Line bars in each column denote the standard errors of three repeated experiments. Different letters represent statistically significant differences at $P = 0.05$ according to the one-way ANOVA test

Although *FfSdhC2* is not directly involved in regulating *F. fujikuroi* growth, we tried to generate a *FfSdhC1* and *FfSdhC2* double mutant in Δ*FfSdhC2* background. While 210 G418-resistant transformants were recovered, all were ectopic mutants, suggesting that *FfSdhC1* and *FfSdhC2* might be essential for *F. fujikuroi* growth and partially functional complementary because the highly efficient homologous integration events in *Fusarium*. To further verify this, we determined the expression of *FfSdhC1* and *-C2* in Δ*FfSdhC2* and Δ*FfSdhC1* by real-time PCR, and the results revealed that expression of *FfSdhC2* in Δ*FfSdhC1* was increased more than three hundred-fold; while expression of *FfSdhC1* in the Δ*FfSdhC2* was similar to that in the wild-type JS16 (Fig. 2c).

Sensitivity of the *FfSdh* deletion mutants to SDHIs and other fungicides

Five different chemical groups of SDHIs, including thifluzamide, boscalid, pydiflumetofen, penflufen, and fluopyram exhibited variable activity against *F. fujikuroi*. While pydiflumetofen exhibited very high inhibitory activity against JS16, thifluzamide and boscalid at 10 μg/mL did not show obvious inhibitory activity against JS16 (Additional file 1: Figure S4a, b), and penflufen and fluopyram inhibited JS16 growth to a lesser extent. Compared to the wild-type, Δ*FfSdhC2* did not show detectable changes in sensitivity to pydiflumetofen, penflufen, and fluopyram. However, Δ*FfSdhA*, *-B*, *-C1*, *-D* significantly reduced sensitivity to these three SDHIs (Fig. 3a, b). In addition, the *FfSdh* mutants did not change their sensitivity to other groups of fungicides dramatically, including the benzimidazole fungicide carbendazim, triazole fungicide tebuconazole, phenylpyrrole fungicide fludioxonil and cyanoacrylate fungicide phenamacril (Fig. 3c, d). To further verify this finding, we determined expression patterns of *FfSdh* genes under a SDHI treatment by real-time PCR assay. As shown in Fig. 3e, expression levels of *FfSdhA*, *B*, *C1*, and *D* were up-regulated 2–4-fold after the wild-type JS16 was treated with 1.5 μg/mL (EC_{90}) pydiflumetofen for 4 h as compared to those of non-treatment, but the expression level of *FfSdhC2* was up-regulated by 7 to 10-fold. These results indicate that FfSdh subunits may be specifically associated with the sensitivity to SDHIs but not other groups of fungicides.

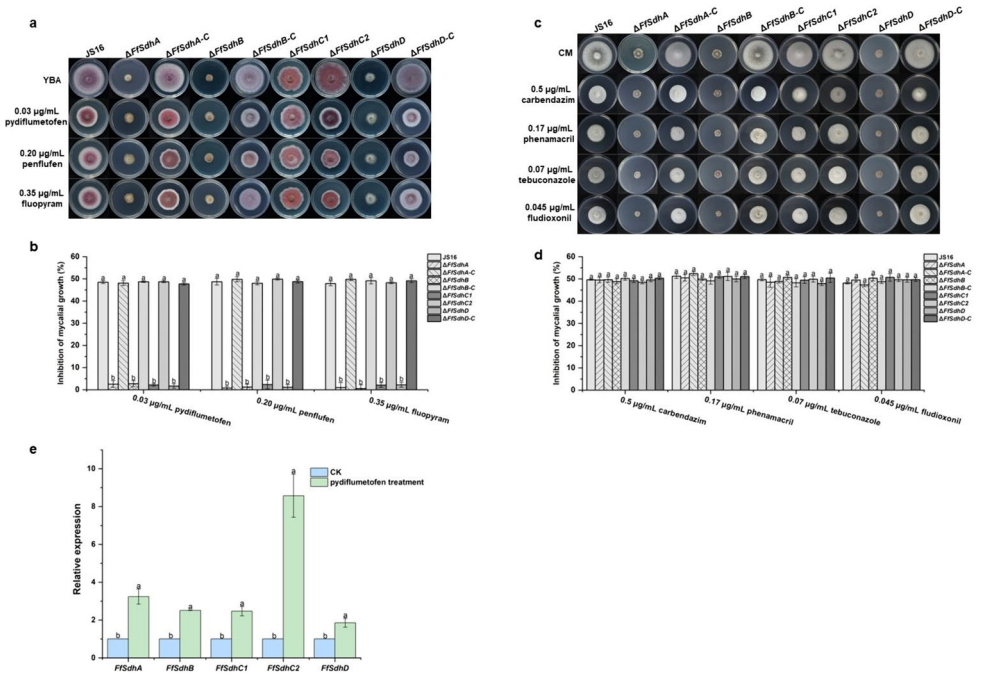

Fig. 3 Comparisons in sensitivity to different fungicides at EC_{50} concentration among the wild-type JS16 and the *FfSdh* deletion mutants. **a** Mycelial growth phenotypes of the wild-type JS16, the *FfSdh* gene deletion mutants, and the complementation strains treated with SDHIs, including pydiflumetofen, penflufen, and fluopyram. The strains were all incubated on YBA at 25℃ for 7 days. **b** Inhibition rate of pydiflumetofen, penflufen, and fluopyram against each strain after 7 days of incubation. Line bars in each column denote the standard errors of three repeated experiments. Different letters represent statistically significant differences for each fungicide at $P = 0.05$ according to the one-way ANOVA test. **c** Mycelial growth phenotypes of the wild-type JS16, the *FfSdh* gene deletion mutants, and the complementation strains treated with non-SDHI fungicides. The strains were incubated on CM at 25℃ for 7 days. **d** Inhibition rate of non-SDHIs against each strain. Line bars in each column denote the standard errors of three repeated experiments. Different letters represent statistically significant differences for each fungicide at $P = 0.05$ according to the one-way ANOVA test. **e** Relative transcription levels of the *FfSdh* gene in JS16 after pydiflumetofen treatment. Each strain was cultured in YEPD lipid medium for 12 h and then treated with pydiflumetofen (1.5 μg/mL) for 4 h. *FfACTIN* was used as an internal control. Different letters represent statistically significant differences for each fungicide at $P = 0.05$ according to the one-way ANOVA test

Sensitivity of the *FfSdh* deletion mutants to various stresses

As shown in Fig. 4a, the *FfSdhC1* and *-C2* deletion mutants, the complementation strain Δ*FfSdhA-C*, Δ*FfSdhB-C*, and Δ*FfSdhD-C* were morphologically indistinguishable from the wild-type isolate JS16 on CM plates supplemented with NaCl (osmotic stress), Congo red (cell wall stress), $CaCl_2$, and oxidative stresses. However, the Δ*FfSdhA*, *-B*, and *-D* mutants showed enhanced sensitivity to $CaCl_2$ and were also hypersensitive to H_2O_2 (Fig. 4b). These results indicate that *FfSdhA*, *B*, and *D* are involved in regulating calcium and oxidative stresses in *F. fujikuroi*.

Involvement of the *FfSdh* in sporulation

Conidia of *F. fujikuroi* have an important role in the disease cycle of rice bakanae disease. As shown in Fig. 5, there was no significant difference in conidiation between Δ*FfSdhC2* and the wild-type isolate JS16. However, the Δ*FfSdhA*, *-B*, and *-D* mutants did not produce conidia at all in the tested condition. Compared to JS16, Δ*FfSdhC1* showed a decrease in conidial production. These results indicate that *FfSdhA*, *B*, and *D* play a critical role in conidiation and that *FfSdhC1* is also involved in the regulation of sporulation in *F. fujikuroi*.

Virulence of the *FfSdh* deletion mutants

The wild-type JS16, Δ*FfSdhC1*, *-C2*, and the complementation strains caused typical symptoms of RBD, and the rice seedlings inoculated with these strains were significantly longer than those of the non-inoculated control, while the Δ*FfSdhA*, *-B*, and *-D* mutants significantly reduced in virulence (Fig. 6a, b). This result showed that *FfSdhA*, *B*, and *D* are indispensable for the virulence of *F. fujikuroi*.

Discussion

In the current study, five *SDH* genes (*FfSdhA*, *B*, *C1*, *C2*, and *D*) were identified in *F. fujikuroi*. Phenotypic assays for the *FfSdh* gene deletion mutants showed that *FfSdhA*, *B*, and *D* play critical roles in hyphal growth, conidiation, and virulence in *F. fujikuroi*. Meanwhile, the

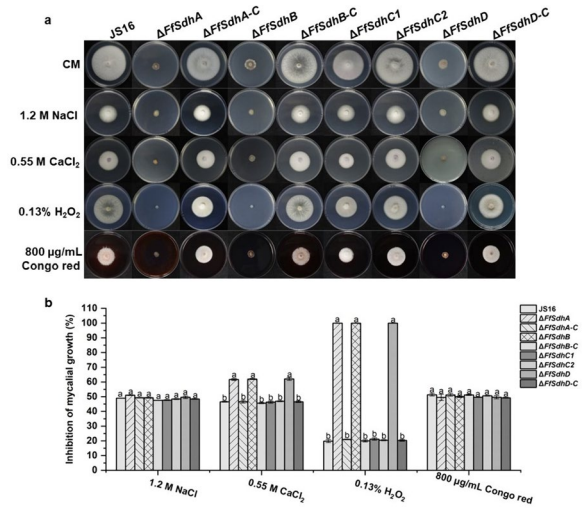

Fig. 4 Sensitivity of the *FfSdh* gene deletion mutants to various stresses. **a** Mycelial growth phenotypes of the wild-type JS16, the *FfSdhA*, *B*, *C1*, *C2*, and *D* gene deletion mutants and the complementation strains under various stress conditions. **b** Inhibition rate of mycelial growth by different stresses. Line bars in each column denote the standard errors of three repeated experiments. Different letters represent statistically significant differences for each stress at $P=0.05$ according to the one-way ANOVA test

FfSdhA, *B*, and *D* gene deletion mutants were hypersensitive to H_2O_2, which can be produced by host plants in response to fungal infection (Shao et al. 2016). Furthermore, the *FfSdhA*, *B*, and *D* deletion mutants exhibited resistance to all SDHIs tested, including pydiflumetofen, penflufen, and fluopyram. The occurrence of fungicide resistance is usually accompanied by fitness costs, which may influence resistance build-up in the absence of fungicide selection pressure (Liu et al. 2022). Thus, phenotypic characterization of Δ*FfSdhA*, *-B*, and *-D* are useful in evaluating the role of *FfSdhA*, *B*, and *D* in the development of SDHIs resistance. In addition, we also found that deletion of *FfSdhC1* led to slightly decreased mycelial growth and sporulation, while the *FfSdhC2* deletion mutant showed similar mycelial growth, conidation, and virulence. However, we could not obtain a double mutant of *FfSdhC1+C2*, similar to those reported in *F. graminearum* (Sun et al. 2023). These results suggest that the *FfSdhC1* and *C2* genes are partial functional complementarity in *Fusarium* spp..

It has been proposed that the paralogous genes might have evolved through duplication from an ancestral gene followed by functional specialization under certain stresses (Magadum et al. 2013). In general, paralogous genes resulting from gene duplication are a powerful source of genetic novelty and provide new substrates for evolution (Iohannes and Jackson 2023). However, the functions of gene duplication in fungicide resistance

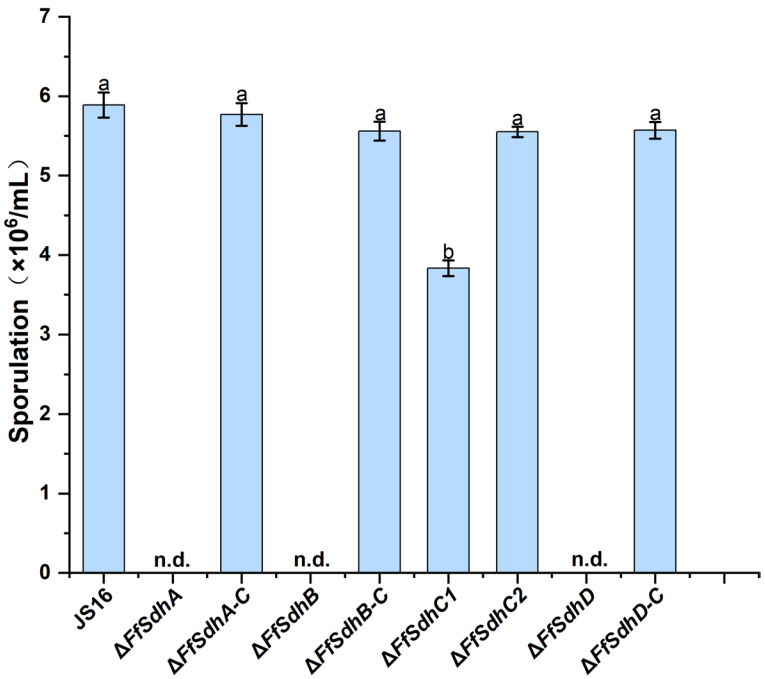

Fig. 5 Sporulation of the wild-type JS16, the *FfSdh* gene deletion mutants, and the complementation strains Δ*FfSdhA-C*, *FfSdhB-C*, and *FfSdhD-C*. Line bars in each column denote standard errors of three repeated experiments. "n.d." represents no conidia detected. Different letters represent statistically significant differences at $P=0.05$ according to the one-way ANOVA test

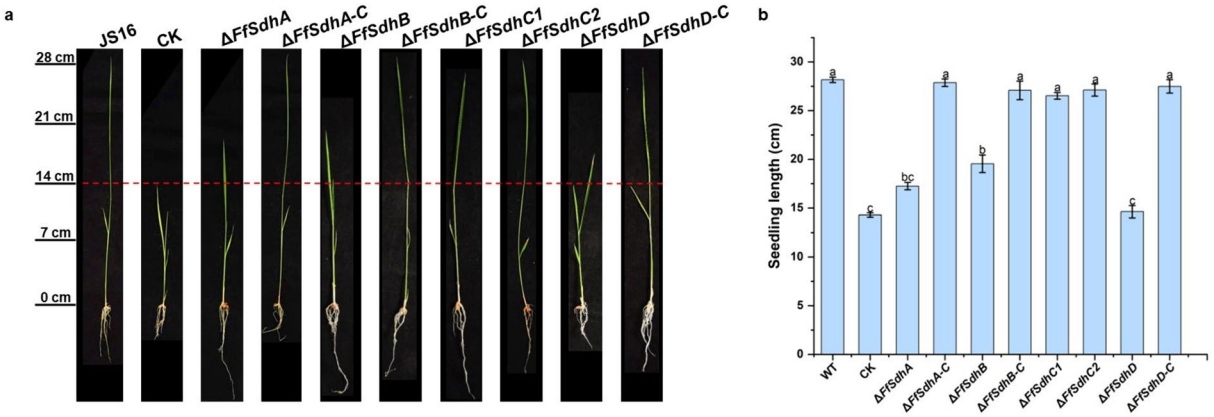

Fig. 6 Pathogenicity assay for the *FfSdh* gene deletion mutants. **a** Symptoms caused by the *FfSdh* gene deletion mutants on rice seedlings. **b** The length of rice seedlings inoculated with the JS16 or each of the *FfSdh* deletion mutants. CK indicates the seedling length of rice without inoculation. Line bars in each column denote the standard errors of three repeated experiments. Different letters represent statistically significant differences at $P = 0.05$ according to the one-way ANOVA test

are largely unknown. In this study, we observed that the expression of *FfSdhC2* in Δ*FfSdhC1* was increased dramatically, which may lead to resistance of Δ*FfSdhC1* to SDHIs. Recently, Steinhauer et al. found that some *Zymoseptoria tritici* isolates are naturally resistant to the stretched heterocycle amide group of SDHIs, which is caused by a gene paralog of *SdhC* (termed *ZtSdhC3*) encoding for an alternative C subunit of succinate dehydrogenase (alt-SdhC). The alt-SdhC associates with the three other Sdh subunits, leading to a fully functional enzyme. Importantly, a unique Qp-site residue within the alt-SdhC protein confers SHA-SDHIs resistance. Moreover, transposon insertions in the promoter of *ZtSdhC3* are also associated with the most robust resistance phenotypes. These results establish that a dispensable paralogous gene determines SDHI fungicide resistance in natural pathogen populations (Steinhauer et al. 2019). Thus, it would be interesting to understand the roles of target paralogs in the sensitivity of many other fungal pathogens to fungicides.

In the current study, we found that the *FfSdhC2* mutant did not alter its sensitivity to SDHIs. However, the *FfSdhC1* mutant showed increased tolerance to SDHIs but not to other groups of fungicides. These results suggest that *FfSdhC1*, but not *C2*, might play a critical role in regulating SDHI sensitivity in *F. fujikuroi.* In line with this finding, Sun and colleagues found that as an FgSdhC paralog, FgSdhC1 is responsible for the natural resistance or less sensitivity of *F. graminearum* to a majority of SDHI fungicides (Sun et al. 2023), suggesting that the dispensable paralogous gene *SdhCs* may play an important role in mediating SDHI resistance in *Fusarium* populations. To date, 24 SDHIs have been launched in the market for controlling various fungal diseases. Since different SDHIs may target different SDH complexes, it is reasonable to believe that cross-reactivity patterns are complex among SDHIs. We expect that this finding will trigger more research on the development of SDHI resistance in pathogenic *Fusarium* and other fungal species.

Conclusions

In this study, we identified five *Sdh* genes in *F. fujikuroi* and carried out a gene deletion for *FfSdhA*, *B*, *C1*, *C2*, and *D*. Through phenotypic assays, we found that *FfSdhA*, *B*, *C1*, and *D* plays critical roles in regulating fungal growth and sensitivity to SDHIs. Although the *FfSdhC2* deletion mutant did not show detectable phenotypic changes as compared to the wild-type strain, we failed to obtain a double mutant of *FfSdhC1+C2*, indicating that the two paralogous *SdhC* genes might be partially functional complementary, and are required for fungal growth in *F. fujikuroi*. The findings of this study provide useful information for enhancing our understanding of the functions of Sdh in pathogenic fungi.

Methods

Strain, media, and fungicides

The *F. fujikuroi* isolate JS16 collected from Zhejiang, China, was used as a wild-type strain for gene deletion experiments. This strain and target gene deletion mutants were grown on potato dextrose agar (PDA) (200 g potato, 20 g dextrose, 18 g agar, and 1 L water) or minimal medium (MM) (10 mM K_2HPO_4, 10 mM KH_2PO_4, 4 mM $(NH_4)_2SO_4$, 2.5 mM NaCl, 2 mM $MgSO_4$, 0.45 mM $CaCl_2$, 9 mM $FeSO_4$, 10 mM glucose, 10 g agar, and 1 L water, pH 6.9) for mycelial growth assay. The completed medium (CM) (10 g glucose, 2 g peptone, 1 g yeast extract, 1 g casamino acids, 6 g $NaNO_3$, 0.52 g KCl,

0.52 g $MgSO_4 \cdot 7\ H_2O$, 1.52 g KH_2PO_4, 1 mL trace elements, 0.01% of vitamins, 10 g agar, and 1 L water, pH 6.5) (Correll et al. 1987) was used for non-SDHI fungicide sensitivity assay. Yeast bacteriological peptone agar medium (YBA) (10 g bacteriology peptone, 10 g yeast extract, 20 g sodium acetate, and 15 g agar in 1 L water) was used to determine the sensitivity of *F. fujikuroi* to SDHIs (Ishii et al. 2016). Yeast extract peptone dextrose medium (YEPD) (10 g peptone, 3 g yeast extract, 20 g D-glucose, made up to 1 L, pH 7.0) was used for spore production measurement.

Penflufen (22.4% active ingredient) and fluopyram (41.7%) were provided by Bayer Crop Sciences Co. Pydiflumetofen (200 g/L), boscalid (50%), and thifluzamide (98%) were obtained from Syngenta, BASF and Zhejiang Hangzhou Yulong Chemical Co., respectively. Thifluzamide was dissolved in methanol to obtain a stock solution of 5 mg of active ingredient/mL.

Verification of *Sdh* introns and analysis of *Sdh* gene expression in *F. fujikuroi*

F. graminearum contains five putative succinate dehydrogenase subunits (*FgSdhA*, *B*, *C*, and *D* with accession No. FGRAMPH1_01G24629, FGRAMPH1_01G18325, FGRAMPH1_01G04785, FGRAMPH1_01G28127, and FGRAMPH1_01G01879 in the FungiDB, respectively). Based on these five putative*FgSdhs*, we obtained the *FfSdhA*, *FfSdhB*, *FfSdhC1*, *FfSdhC2*, and *FfSdhD* from *F. fujikuroi* genome by homologous comparison. *FfSdhA*, *B*, *C1*, *C2*, and *D* were predicted to possess five, two, two, two, and two introns, respectively. To verify the existence and size of these predicted introns, RNA was extracted from the mycelia of the wild-type isolate JS16 of *F. fujikuroi* using TRIzol (TaKaRa Biotechnology, Dalian, China) according to the manufacturer's instructions. The first strand of cDNA was synthesized using 1 μg of total RNA per sample by HiScript II Q RT SuperMix kit (R223–01, Vazyme, Nanjing, China) according to the manufacturer's instructions. PCR amplifications were performed with the primer pair A-F + A-R, B-F + B-R, C1-F + C1-R, C2-F + C2-R, and D-F + D-R (Additional file 2: Table S1) for *FfSdhA, B, C1, C2*, and *D*, respectively, using the JS16 cDNA and DNA as templates.

Relative expression levels of *FfSdh* genes were measured by real-time PCR assay. Briefly, each strain was cultured in 100 mL YEPD liquid medium for 12h at 25°C. Then, fresh mycelia were harvested for total RNA extraction and cDNA synthesis. Expression levels of *Sdh* genes were determined with qRT-PCR using HiScript II QRT SuperMix (Vazyme Biotech, Nanjing, China). For each sample, PCR amplifications with primer pair Ff-actin-F + Ff-actin-R were used as the endogenous control. The primers used for qRT-PCR assays are listed in Additional file 2: Table S1. The relative expression levels of target genes were calculated using the $2^{-\Delta\Delta Ct}$ method (Kl 2001). The experiments were independently performed three times.

Phylogenic analysis of SdhC proteins in *F. fujikuroi* and *Fusarium* spp.

The sequence identities of *FfSdhC1* and *FfSdhC2* were analyzed using the DNAMAN software, and the locations of the transmembrane regions of *FfSdhC1* and *FfSdhC2* were analyzed using the DeepTMHMM (https://dtu.biolib.com/DeepTMHMM). Localization of *FfSdhC1* and *FfSdhC2* was predicted by using the WoLF PSORT (https://wolfpsort.hgc.jp/). *Fusarium* spp. orthologs of *FfSdhC1* and *FfSdhC2* were retrieved in ENSEMBL BLAST (https://fungi.ensembl.org/Multi/Tools/Blast), respectively. The identity of *SdhC* orthologues from *Fusarium* spp. or non *Fusarium* spp. was analyzed by the DNAMAN software.

Generation of deletion and complementation mutants

The target gene deletion mutants Δ*FfSdhA*, *-B*, *-C1*, *-C2*, and *-D* were constructed using a protocol described previously (Yun et al. 2014). Briefly, the open reading frame (ORF) of *FfSdhs* was replaced with hygromycin (*HPH*). Transformation for *F. fujikuroi* was performed via the polyethyleneglycol (PEG) mediated protoplast transformation method (Proctor et al. 1995). For Δ*FfSdhA*, *-B*, and *-D* complementation, ORFs of *FfSdhA*, *B*, and *D* fused with a geneticin (*G418*) resistance cassette were introduced into the corresponding mutant. To construct the Δ*FfSdhC1+C2* dual-deletion mutant, the *FfSdhC1* gene was tried to be knocked out in the Δ*FfSdhC2* using geneticin (*G418*) resistance as a selection marker. All of the deletion mutants were verified by PCR with relevant primers listed in Additional file 2: Table S1.

Sensitivity of the *FfSdh* deletion mutants to SDHIs and non-SDHIs fungicides

To determine sensitivity of the *FfSdh* deletion mutants to SDHIs, a mycelial plug (5 mm in diameter) taken from the periphery of a 3-day-old colony was placed on the center of a YBA plate amended with pydiflumetofen at 0.03 μg/mL, penflufen at 0.20 μg/mL, and fluopyram at 0.35 μg/mL, respectively. The concentrations of other fungicides are indicated in the corresponding figures. In addition, to evaluate the sensitivity of the *FfSdh* deletion mutants to non-SDHIs, a mycelial plug (5 mm in diameter) taken from the periphery of a 3-day-old colony was placed in the center of a CM plate amended with carbendazim at 0.5 μg/mL, phenamacril at 0.17 μg/mL, tebuconazole at 0.07 μg/mL or fludioxonil at 0.045 μg/mL, respectively. Two replicates for each concentration were used for each mutant. After incubation at 25°C for 7 days,

the colony diameter in each plate was measured in two perpendicular directions with the original mycelial plug diameter (5 mm) subtracted from each measurement. The experiment was repeated three times.

Stress assay

Phenotypic assay was performed on CM supplemented with NaCl, $CaCl_2$, H_2O_2, or Congo red at various concentrations. Each plate was inoculated with a mycelial plug (5 mm in diameter) taken from the periphery of a 3-day-old colony of each isolate/mutant. After incubation at 25°C for 7 days in the dark, colony diameter was examined for each plate. The experiment was repeated three times.

Sporulation assay

For the sporulation assay, three mycelial plugs (5 mm in diameter) of each isolate/mutant taken from the periphery of a 3-days-old colony were inoculated in a 50 mL flask containing 30 mL of YEPD medium. The flasks were incubated at 25°C for 36 h in a shaker (180 rpm). For each isolate/mutant, the number of conidia in the broth was determined using a hemacytometer. The experiment was repeated three times.

Virulence assay

The RBD susceptible variety Zhongzao 39 was used for the virulence assay. The artificial inoculation of *F. fujikuroi* referred to a previously published protocol (Zhao et al. 2022). Briefly, after sterilization with 3% H_2O_2 for 2 h, rice seeds were further soaked for 2 days and then germinated for 1 day. Each sprouted seed together with a mycelial plug of each *F. fujikuroi* mutant (a thin layer of vermiculite was used to separate seed with mycelial plug) was incubated into a sterilized vermiculite test tube. Five replicates were used for each strain. Samples were then cultured in an incubator with 12 h of light and 12 h of darkness at 30°C. After 15 days of inoculation, the lengths of rice seedlings were examined and photographed. The experiment was repeated three times.

Abbreviations

PCR	Polymerase chain reaction
RBD	Rice bakanae disease
SDH	Succinate dehydrogenase
SDHIs	Succinate dehydrogenase inhibitors

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s42483-024-00254-9.

Additional file 1: Figure S1. Schematic map of gene structures and protein domains of *FfSdhA*, *B*, *C1*, *C2*, and *D*. **a** Gene structures of *FfSdhA*, *B*, *C1*, *C2*, and *D*. **b** Protein domains of FfSdhA, B, C1, C2, and D. **Figure S2.** Alignment of SdhC ortholog proteins from *Fusarium* spp. **a** Alignment of FfSdhC1 (FFUJ_08951) and FfSdhC2 (FFUJ_12139). **b** Alignment of FfSdhC1 orthologs from different *Fusarium* species. **c** Alignment of FfSdhC2 orthologs from different *Fusarium* species. **d** Alignment of FfSdhC1 orthologs from different non *Fusarium* species. **e** Alignment of FfSdhC2 orthologs from different non *Fusarium* species. **Figure S3.** Generation and identification of *F. fujikuroi FfSdh* gene replacement mutants. **a** Schematic representation of the *FfSdh* gene replacement strategy. The upper sketch represents the genomic locus target of the replacement construct. Two gray fragments represent the left and right homologous arms of the *Sdh* gene, respectively. The blue fragment represents the target *FfSdh* genes, and the box arrow represents the hygromycin (HPH) resistant gene that connects with the left and right homologous arms of each *SDH*. The positions of primer binding sites are indicated at the lower panel of each figure. **b** PCR assay to verify recombination. **Figure S4.** Boscalid and thifluzamide at 10 μg/mL don't show obvious inhibitory activity. **a** Colony morphology of the JS16 and *FfSdh* gene deletion mutants cultured on YBA with or without SDHIs at 25℃ for 7 days. **b** Mycelial growth diameter was determined for each strain after 7 days of incubation on YBA, 10 μg/mL boscalid, and 10 μg/mL thifluzamide. Line bars in each column denote the standard errors of three repeated experiments. Different letters represent statistically significant differences for each strain at $P = 0.05$ according to the one-way ANOVA test.

Additional file 2: Table S1. Oligonucleotide primers used in this study and their relevant characteristics.

Acknowledgements

Not applicable.

Authors' contributions

ZM conceived and supervised this project. MF, HQ, and WS performed the experiment. YY, YC, and HZ analyzed the data. MF, ZM, and YZ wrote the manuscript. All authors read and approved the final manuscript.

Funding

This research was supported by the Key Project of National Natural Science Foundation of China (U21A20219), National Key Research and Development Program of China (2022YFD1400100), and China Agriculture Research System (CARS-03-29).

Availability of data and materials

Not applicable.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

These authors declare they have no competing interests.

Received: 17 March 2024 Accepted: 16 May 2024
Published online: 18 July 2024

References

Amatulli MT, Spadaro D, Gullino ML, Garibaldi A. Molecular identification of *Fusarium* spp. associated with bakanae disease of rice in Italy and assessment of their pathogenicity. Plant Pathol. 2010;59:839–44. https://doi.org/10.1111/j.1365-3059.2010.02319.x.

Asmaul H, Asaduzzaman MM, Nor NMIM. Rice bakanae disease: an emerging threat to rice production in Bangladesh. Asian J Med Biol Res. 2021;6(4):608–10. https://doi.org/10.3329/ajmbr.v6i4.51224.

Bashyal BM, Aggarwal R, Sharma S, Gupta S, Rawat K, Singh D, et al. Occurrence, identification and pathogenicity of *Fusarium* species associated

with bakanae disease of basmati rice in India. Eur J Plant Pathol. 2016;144:457–66. https://doi.org/10.1007/s10658-015-0783-8.
Chen Z, Gao T, Liang S, Liu K, Zhou M, Chen C. Molecular mechanism of resistance of *Fusarium fujikuroi* to benzimidazole fungicides. FEMS Microbiol Lett. 2014;357:77–84. https://doi.org/10.1111/1574-6968.12504.
Chen W, Wei L, Zhao W, Wang B, Zheng H, Zhang P, et al. Resistance risk assessment for a novel succinate dehydrogenase inhibitor pydiflumetofen in *Fusarium Asiaticum*. Pest Manag Sci. 2021;77:538–47. https://doi.org/10.1002/ps.6053.
Correll JC, Klittich CJR, Leslie JF. Nitrate non-utilizing mutants of *Fusariumoxysporum* and their use in vegetative compatibility tests. Phytopathology. 1987;77:1640–6.
Fiyaz RA, Yadav AK, Krishnan SG, Ellur RK, Bashyal BM, Grover N, et al. Mapping quantitative trait loci responsible for resistance to bakanae disease in rice. Rice. 2016;9:45. https://doi.org/10.1186/s12284-016-0117-2.
Hou Y, Qu X, Mao X, Kuang J, Duan Y, Song X, et al. Resistance mechanism of *Fusarium fujikuroi* to phenamacril in the field. Pest Manag Sci. 2018;74:607–16. https://doi.org/10.1002/ps.4742.
Iohannes SD, Jackson D. Tackling redundancy: genetic mechanisms underlying paralog compensation in plants. New Phytol. 2023;240:1381–9. https://doi.org/10.1111/nph.19267.
Ishii H, Zhen F, Hu M, Li X, Schnabel G. Efficacy of SDHI fungicides, including benzovindiflupyr, against *Colletotrichum* species. Pest Manag Sci. 2016;72:1844–53. https://doi.org/10.1002/ps.4216.
Kim SH, Park MR, Kim YC, Lee SW, Choi BR, Lee SW, et al. Degradation of prochloraz by rice bakanae disease pathogen *Fusarium fujikuroi* with differing sensitivity: a possible explanation for resistance mechanism. J Korean Soc Appl Biol Chem. 2010;53:433–9. https://doi.org/10.3839/jksabc.2010.067.
Kl L. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods. 2001;25:402–8.
Leroux P, Gredt M. Evolution of fungicide resistance in the cereal eyespot fungi *Tapesia yallundae* and *tapesia acuformis* in France. J Pestic Sci. 1997;51:321.
Liu K, Wen Z, Ma Z, Shao W. Biological and molecular characterizations of fluxapyroxad-resistant isolates of Botrytis Cinerea. Phytopathol Res. 2022;4:1–11. https://doi.org/10.1186/s42483-022-00107-3.
Li Y, Tang Y, Xue Z, Wang Y, Shi Y, Gao X, et al. Resistance risk and resistance-related point mutation in SdhB and SdhC1 of cyclobutrifluram in *Fusarium pseudograminearum*. J Agric Food Chem. 2023;71:1886–95. https://doi.org/10.1021/acs.jafc.2c08022.
Liu Y, Sun Y, Bai Y, Cheng X, Li H, Chen X, et al. Study on mechanisms of resistance to SDHI fungicide pydiflumetofen in *Fusarium fujikuroi*. J Agric Food Chem. 2023;71:14330–41. https://doi.org/10.1021/acs.jafc.3c03678.
Magadum S, Banerjee U, Murugan P, Gangapur D, Ravikesavan R. Gene duplication as a major force in evolution. J Genet. 2013;92:155–61. https://doi.org/10.1007/s12041-013-0212-8.
Moosavi B, Berry EA, Zhu XL, Yang WC, Yang GF. The assembly of succinate dehydrogenase: a key enzyme in bioenergetics. Cell Mol Life Sci. 2019;76:4023–42. https://doi.org/10.1007/s00018-019-03200-7.
Proctor RH, Hohn TM, McCormick SP. Reduced virulence of *Gibberella zeae* caused by disruption of a trichothecene toxin biosynthetic gene. Mol Plant Microbe Interact. 1995;8:593–601.
Shao W, Yang Y, Zhang Y, Lv C, Ren W, Chen C. Involvement of BcStr2 in methionine biosynthesis, vegetative differentiation, multiple stress tolerance and virulence in *Botrytis cinerea*. Mol Plant Pathol. 2016;17:438–47. https://doi.org/10.1111/mpp.12292.
Shao W, Wang J, Wang H, Wen Z, Liu C, Zhang Y, et al. *Fusarium graminearum* FgSdhC1 point mutation A78V confers resistance to the succinate dehydrogenase inhibitor pydiflumetofen. Pest Manag Sci. 2022;78:1780–8. https://doi.org/10.1002/ps.6795.
Steinhauer D, Salat M, Frey R, Mosbach A, Luksch T, Balmer D, et al. A dispensable paralog of succinate dehydrogenase subunit C mediates standing resistance towards a subclass of SDHI fungicides in *Zymoseptoria tritici*. PLOS Pathog. 2019;15:e1007780. https://doi.org/10.1371/journal.ppat.1007780.
Sun H, Cai S, Liu H, Li X, Deng Y, Yang X, et al. FgSdhC paralog confers natural resistance toward SDHI fungicides in *Fusarium graminearum*. J Agric Food Chem. 2023;71:20643–53. https://doi.org/10.1021/acs.jafc.3c06288.
Sun Y, Liu Y, Zhou L, Liu X, Wang K, Chen X, et al. Activity of fungicide cyclobutrifluram against *Fusarium fujikuroi* and mechanism of the pathogen resistance associated with point mutations in FfSdhB, FfSdhC2 and FfSdhD. J Integr Agric. 2024. https://doi.org/10.1016/j.jia.2024.01.004.
Sun Y, Shi H, Mao C, Wu J, Zhang C. Activity of a SDHI fungicide penflufen and the characterization of natural-resistance in *Fusarium fujikuroi*. Pestic Biochem Physiol. 2021;179:104960. https://doi.org/10.1016/j.pestbp.2021.104960.
Wu J, Mao H, Sun Y, Wei F, Wang H, Zhang C. Treatment with succinate dehydrogenase inhibitor Y12196 protects strawberries from boscalid-resistant *Botrytis cinerea* with the H272R mutation in SDHB. Phytopathol Res. 2023;5:22. https://doi.org/10.1186/s42483-023-00173-1.
Xue Z, Zhong S, Shen J, Sun Y, Gao X, Wang X, et al. Multiple mutations in SDHB and SDHC2 subunits confer resistance to the succinate dehydrogenase inhibitor cyclobutrifluram in *Fusarium fujikuroi*. J Agric Food Chem. 2023;71:3694–704. https://doi.org/10.1021/acs.jafc.2c08023.
Yin Y, Liu X, Li B, Ma Z. Characterization of sterol demethylation inhibitor-resistant isolates of *Fusarium asiaticum* and *F. graminearum* collected from wheat in China. Phytopathology. 2009;99:487–97. https://doi.org/10.1094/PHYTO-99-5-0487.
Yun Y, Liu Z, Zhang J, Shim WB, Chen Y, Ma Z. The MAPKK FgMkk1 of *Fusarium graminearum* regulates vegetative differentiation, multiple stress response, and virulence via the cell wall integrity and high-osmolarity glycerol signaling pathways. Environ Microbiol. 2014;16:2023–37. https://doi.org/10.1111/1462-2920.12334.
Zhang C, Chen Y, Yin Y, Ji H, ShimWB, Hou Y, et al. A small molecule species specifically inhibits *Fusarium* myosin I: JS399-19 inhibits fungal myosin I. Environ Microbiol. 2015;17:2735–46. https://doi.org/10.1111/1462-2920.12711.
Zhao K, Liu L, Huang S. Genome-wide identification and functional analysis of the bZIP transcription factor family in rice bakanae disease pathogen, *Fusarium fujikuroi*. Int J Mol Sci. 2022;23:6658. https://doi.org/10.3390/ijms23126658.