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Cytochrome *c* Oxidase Influences Pyraclostrobin Sensitivity in *Fusarium graminearum* by Regulating *FgAox* Through Transcription Factors FgAod2 and FgAod5

Zhiwen Wu, Furong Chen, Lingrong Zhang, Mingguo Zhou, and Yiping Hou*

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ABSTRACT: Cytochrome *c* oxidase (Cox) is a crucial terminal oxidase in the electron transport chain. In this study, we generated 14 Cox gene deletion or overexpression mutants in *Fusarium graminearum*. Fungicide sensitivity tests revealed that 11 Cox gene deletion mutants displayed resistance to pyraclostrobin, while 10 overexpression mutants showed hypersensitivity. RNA-Seq and RT-qPCR analyses demonstrated the upregulation of *FgAox* (alternative oxidase in *F. graminearum*), *FgAod2*, and *FgAod5* (alternative oxidase deficiency in *F. graminearum*) in ΔFgCox4−2 and ΔFgCox17−75 mutants. In 11 Cox gene deletion mutants, *FgAox* expression was significantly upregulated, whereas in 10 Cox gene overexpression mutants, it was significantly downregulated. *FgAox* overexpression mutants exhibit resistance to pyraclostrobin, while *FgAox* deletion mutants show hypersensitivity to pyraclostrobin. FgAod2 and FgAod5 were identified as transcription factors for *FgAox*. Our findings reveal that *FgCox* influences pyraclostrobin sensitivity by regulating *FgAox* through FgAod2 and FgAod5. Understanding pyraclostrobin resistance mechanisms in *F. graminearum* could help develop better fungicide rotation and application strategies to manage resistance and guide the creation of new fungicides targeting different pathways.

KEYWORDS: *cytochrome c oxidase, Fusarium graminearum, pyraclostrobin, resistance mechanism, alternative oxidase*

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1. INTRODUCTION

Wheat is a vital global food crop, ranking among the top three alongside corn and rice, and plays a crucial role in ensuring food security worldwide.[1,2] With a global sown area of 217 million hectares and a total production of 752 million tons in 2019−2020, wheat is relied upon by approximately 40% of the world's population as a staple food.[3−5] However, wheat production faces numerous challenges, with Fusarium head blight (FHB) being a significant threat.[6] FHB outbreaks can lead to devastating yield losses exceeding 70%,[7,8] and the pathogenic fungus *Fusarium graminearum* responsible for FHB also produces mycotoxins such as deoxynivalenol, trichothecenes, and zearalenone.[9] These mycotoxins pose severe health risks, including vomiting, gastrointestinal diseases, and even death in severe cases.[10−12]

Up to now, the main method for controlling *F. graminearum* has been the use of fungicides. According to the data of FRAC Code List 2023, many fungicides target various components of the mitochondrial respiratory electron-transport chain (ETC), such as complexes I (diflumetorim), II (pydiflumetofen), III (pyraclostrobin), and V (fluazinam). However, the specific targeting of complex IV by fungicides has not been extensively studied or reported, and its regulating effect on the sensitivity of fungicides used to control FHB remains unclear. Therefore, research of the role of complex IV in *F. graminearum* can enhance our understanding of its function and its impact on fungicide sensitivity.

Cytochrome *c* oxidase (Cox), also known as complex IV, is a multisubunit complex located in the inner mitochondrial membrane. It plays a critical role in regulating the redox state of mitochondria, oxidative phosphorylation, and the overall cellular redox balance.[13,14] Cox acts as a terminal oxidase in the ETC, transferring electrons from reduced cytochrome *c* to oxygen and resulting in the production of water molecules. Additionally, this process generates an intracellular proton gradient, which is utilized by proton transmembrane transport to produce ATP.[15,16] Extensive research on Cox has been conducted in various fields such as medicine, botany, and entomology.[17−20] Especially in the medical field, drug target development and design for Cox have been investigated extensively. For example, Cox inhibitors like ADDA 5 hydrochloride have been identified and studied for their potential therapeutic usage.[21] However, there are relatively few reports on Cox research in plant pathogenic fungi. The popularity of Cox in the medical field also indicates the rationality and necessity of its research purpose in plant pathogenic fungi.

In this study, we identified 17 Cox genes in *F. graminearum* using the BLASTP method, referencing the Cox protein sequence of *Saccharomyces cerevisiae* from the SGD (Saccharomyces Genome Database).[22] Out of the identified Cox genes, Cox1, Cox2, and Cox3 were found to be encoded by

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mitochondrial DNA, making it impossible to obtain deletion mutants for these genes in our lab. Therefore, we just successfully obtained deletion or overexpression mutants for the remaining 14 *FgCox* genes, which are encoded by nuclear genes, using a homologous replacement strategy. We observed a significant decrease in sensitivity to the QoI (quinone outside inhibitor) fungicide pyraclostrobin in the ΔFgCox10 mutant, while the sensitivity of the other 13 *FgCox* gene deletion mutants to QoI fungicides remains unclear.

According to previous studies, the resistance mechanisms of QoI fungicides mainly fall into two categories: first, point mutations in the target gene *Cytb*, such as the substitution of glycine at position 143 with alanine (G143A), which is associated with stronger resistance[23] and second, the alternative oxidase (Aox) pathway, a branch of the ETC. In this pathway, the key enzyme, Aox, directly transfers electrons to oxygen via coenzyme Q, bypassing complexes III and IV. In *Lasiodiplodia theobromae*, the expression of *Aox* was significantly upregulated by 33-fold, resulting in notable resistance to pyraclostrobin.[24] Similarly, in *Mycosphaerella graminicola*, high Aox expression reduced the pathogen's sensitivity to pyraclostrobin.[25]

Therefore, the objectives of this study are as follows: (i) clarify the role of *FgCox* genes in the sensitivity of *F. graminearum* to pyraclostrobin and (ii) analyze the resistance mechanism of *FgCox* deletion mutants to pyraclostrobin. These findings will enhance our understanding of *FgCox*'s involvement in QoI fungicide sensitivity in *F. graminearum* and shed light on the resistance mechanisms associated with these fungicides.

2. MATERIALS AND METHODS

2.1. Media and Fungicides. PDA (potato dextrose agar) medium was used to incubate the strain.[26] AEA (alkyl ester agar) medium was used to perform the sensitivity test. AEB liquid medium is AEA medium without agar.[25] SNB (synthetic low-nutrient broth) medium was used to obtain the spores.[22] Czapek's medium was used to induce the pyraclostrobin-resistant mutants.[27]

Pyraclostrobin (available ingredient 97.5%) was offered by Jiangsu Xinnong Chemical Co., Ltd. and dissolved in dimethyl sulfoxide (DMSO) to obtain a 10 mg/mL stock solution.

2.2. Isolation of *F. graminearum*. PH-1 was stored in our laboratory, and XX6−35 was collected from a field in the Jiangsu province of China and identified by morphological and ITS assays.

2.3. In Vitro Sensitivity of *FgCox* Deletion and Overexpression Mutants to Pyraclostrobin. The protocol for gene deletion, complementation, and verification was conducted according to the established protocol by Yu and Ge.[28−30] To further evaluate the sensitivity of *FgCox* deletion mutants to QoI fungicide pyraclostrobin, fresh plugs (5 mm) from the margins of *FgCox* deletion mutants, complemented strains, and PH-1 were transferred onto AEA plates (9 cm) supplemented with a series of concentrations of pyraclostrobin (0, 0.0016, 0.008, 0.04, 0.2, 1, and 5 μg/mL). The plates were then incubated at 25 °C in darkness. Control plates with DMSO were included. Each treatment was replicated three times.[31]

Once the diameter of the control reached approximately 6 cm, the diameter of each treatment was measured by using the crisscross method. The inhibition rate of each concentration on mycelial growth of *FgCox* deletion mutants, complemented strains, and PH-1 was calculated using the following formula in Excel: Inhibition rate (%) = (diameter of control − diameter of treatment)/(diameter of control −5 mm) × 100. The EC_{50} values of pyraclostrobin for *FgCox* deletion mutants, complemented strains, and PH-1 were calculated using SPSS 14.0 software based on linear regression of the inhibition rate on log-transformed fungicide concentration.[28] This experiment was performed in triplicate to ensure accuracy and reliability of the results.

To further validate the regulatory role of *FgCox* in the sensitivity of *F. graminearum* to pyraclostrobin, we generated the overexpression mutants of *FgCox* in *F. graminearum* and assessed the sensitivity to pyraclostrobin. The RP27 promoter was added in front of the target gene sequence of the *FgCox*.[32,33] The relative expression levels of *FgCox* overexpression mutants were determined using quantitative real-time PCR (RT-qPCR).[34] The specific cycling conditions of RT-qPCR are shown in Table S2. The sensitivity of *FgCox* overexpression mutants to pyraclostrobin was determined as mentioned above and compared with PH-1. This experiment was conducted in triplicate.

2.4. RNA-Seq Analysis of the *FgCox* Deletion Mutants after Treatment with Pyraclostrobin. In this study, we found that the 11 *FgCox* gene deletion mutants exhibited significantly reduced sensitivity to the QoI fungicide pyraclostrobin, with notable decreases observed in the ΔFgCox4−2 and ΔFgCox17−75 mutants compared to that observed in other mutants. To delve into the resistance mechanisms of these mutants, we conducted RNA-Seq analysis on them alongside the PH-1 strain.[35] Initially, cultures were grown on PDA plates for 3 days and then transferred to SNB medium, where they were shaken for an additional 3 days. Spores (1×10^6 mL^{-1}) were subsequently harvested, washed, and inoculated into 250 mL Erlenmeyer flasks containing 100 mL of AEB and shaken at 25 °C and 175 rpm for 48 h. The resulting mycelium was filtered, collected, and evenly divided into two portions by using electronic scales. Following inoculation, one set of cultures received pyraclostrobin at EC_{50} concentrations, while the other set served as the control. After 6 h of incubation, mycelium samples were harvested, processed, and promptly sent for RNA-Seq analysis at Shanghai Bioprofile Biotechnology Co., Ltd.

2.5. Relative Expression Levels of *FgAox* in *FgCox* Deletion or Overexpression Mutants. According to the results of RNA-Seq, we found that the expression level of the Aox (*FgAox*, FGSG_01342) was significantly increased in ΔFgCox4−2 and ΔFgCox17−75 mutants.

To determine the expression level of the *FgAox* in all *FgCox* deletion and overexpression mutants, we used the RT-qPCR method. The mycelium from each strain was treated with pyraclostrobin at an EC_{50} concentration specific to each strain. The control groups were treated with DMSO. Each treatment was replicated three times. RNA extraction was performed using the Steady Pure RNA Extraction Kit [Accurate Biotechnology (Hunan) Co., Ltd., China]. Subsequently, cDNA synthesis was carried out using the Evo M-MLV RT Mix Kit [Accurate Biotechnology (Hunan) Co., Ltd., China]. The housekeeping gene GAPDH (glyceraldehyde-3-phosphate dehydrogenase, FGSG_06257) was employed for normalization purposes. Gene expression analysis: Relative gene expression levels were analyzed using the threshold cycle ($2^{-\Delta\Delta CT}$) method.[36] The Quantity Studio 6 Flex software was utilized to perform this analysis.

2.6. Sensitivity Test of *FgAox* Deletion and Overexpression Mutants to Pyraclostrobin. To further investigate the role of *FgAox* in the sensitivity of *F. graminearum* to pyraclostrobin, we generated the *FgAox* deletion mutants, complemented strain, and overexpression mutants. The methods for gene deletion, complementation, overexpression, and verification were conducted according to the protocol established by Yu and Ge.[28,29]

To assess the sensitivity of these mutants to pyraclostrobin, we performed a similar sensitivity test to that mentioned before. The concentrations of pyraclostrobin used in this experiment were 0, 0.0009765625, 0.0039625, 0.015625, 0.0625, 0.25, and 1 μg/mL. This experiment was conducted in triplicate to ensure the accuracy and reliability of the results.

2.7. Relative Expression Levels of *FgAod2* and *FgAod5* in *FgCox* Deletion Mutants. Our RNA-Seq results indicated that genes differentially expressed in ΔFgCox4−2 and ΔFgCox17−75 mutants were enriched in the glycolysis/gluconeogenesis pathways. Notably, *FgAod2* and *FgAod5* were identified as key genes involved in this pathway based on the KEGG database (Kyoto Encyclopedia of Genes and Genomes). So, to investigate the relative expression levels of *FgAod2* and *FgAod5* in the *FgCox* deletion mutants, we randomly selected six *FgCox* deletion mutants (ΔFgCox4, ΔFgCox7C,

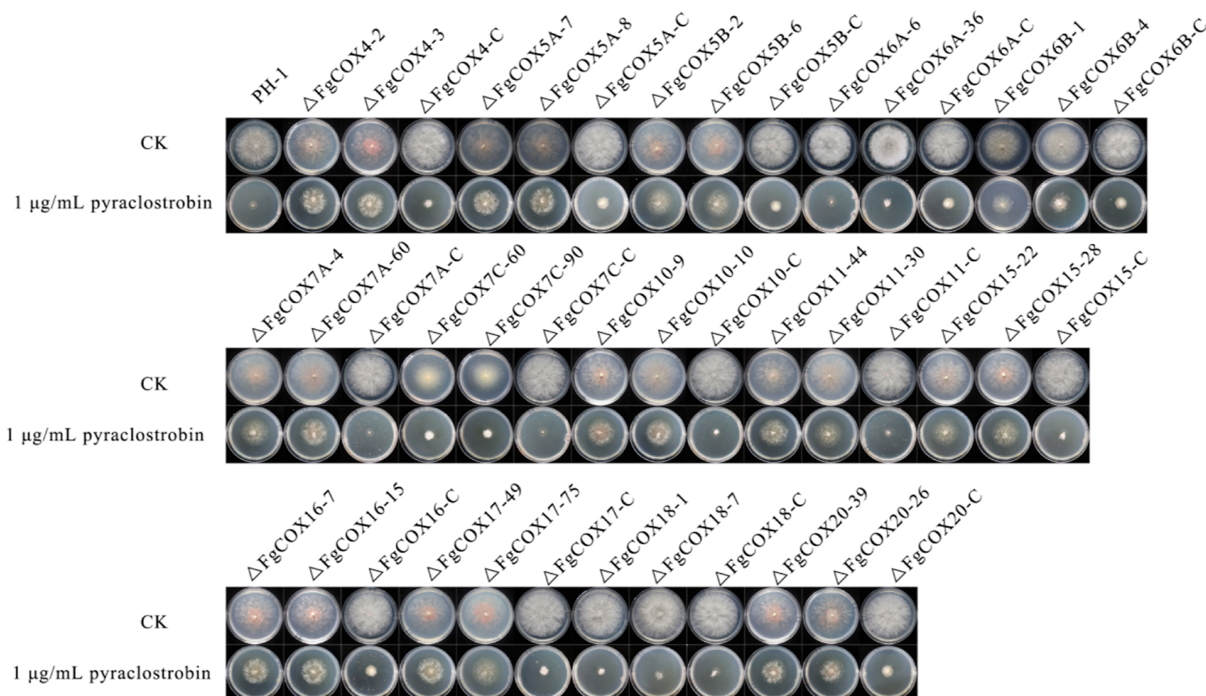

Figure 1. Sensitivity of *FgCox* deletion mutants to QoI fungicide pyraclostrobin.

ΔFgCox10, ΔFgCox15, ΔFgCox17, and ΔFgCox18), along with their complemented strain and wild-type strain PH-1 to determine the relative expression levels of *FgAod*2 and *FgAod*5. This experiment was conducted in triplicate to ensure accuracy and reproducibility.

2.8. Sensitivity Test of *FgAod*2 and *FgAod*5 Deletion and Overexpression Mutants to Pyraclostrobin. To gain further insights into the role of *FgAod*2 and *FgAod*5 in the sensitivity of *F. graminearum* to pyraclostrobin, we generated deletion and overexpression mutants for *FgAod*2 and *FgAod*5 in *F. graminearum*. The sensitivity of these mutants to pyraclostrobin was then determined by following the aforementioned protocol. Again, this experiment was conducted in triplicate to obtain robust and consistent results.

2.9. In Vitro Selection for Pyraclostrobin-Resistant Mutants in *F. graminearum*. To further determine the regulated effect of *FgAox*, *FgAod*2, and *FgAod*5 in pyraclostrobin-resistant mutants, we induced the mutants using two wild-type strains: PH-1 and XX6−35. The fungicide-taming method was employed for this purpose.[35] Fresh plugs were selected from the colony edge of PH-1 or XX6−35, respectively, and transferred onto Czapek's medium amended with 2000 μg/mL pyraclostrobin. Each plate had seven plugs, and each strain was transferred onto 300 plates. After being cultured at 25 °C in darkness for 1 month, the scalloped horns were picked out and transferred onto PDA plates set as putative-resistant mutants. The sensitivity of these putative-resistant mutants to pyraclostrobin was determined as mentioned earlier. To determine the resistance stability, all resistant mutants were activated on PDA plates and transferred onto a new PDA plate after 24 h. This process was repeated 10 times, and the sensitivity of the 10th-generation resistant mutants to pyraclostrobin was determined again. This experiment was conducted in triplicate to ensure the reliability of the results.

2.10. Relative Expression Levels of *FgAox*, *FgAod*2, and *FgAod*5 in Pyraclostrobin-Resistant Mutants. To further determine the relative expression levels of *FgAox*, *FgAod*2, and *FgAod*5 in pyraclostrobin-resistant mutants, the mycelium of resistant mutants and wild-type strains (PH-1 and XX6−35) was treated with pyraclostrobin (at the EC_{50} concentration specific to each strain). After treatment, RNA extraction was performed using the same method mentioned earlier, and the relative expression levels of *FgAox*, *FgAod*2, and *FgAod*5 were determined through RT-qPCR analysis. This experiment was conducted in triplicate to ensure the reliability of the results.

2.11. Yeast One-Hybrid Assay. According to previous research, Aod2 and Aod5 were the transcription factors of *Aox* in *Neurospora crassa*.[37,38] In the Fungal Transcription Factor Database (FTFD, http://ftfd.snu.ac.kr),[39] we found that both *FgAod*2 (locus name FGSG_01562T0) and *FgAod*5 (locus name FGSG_08626T0) are transcription factors containing the zinc finger protein (ZN2CYS6) domain in *F. graminearum*. To investigate the relationship between *FgAox*, *FgAod2*, and *FgAod5*, we employed a yeast one-hybrid (Y1H) assay. The cDNA sequence of *FgAod*2/*FgAod*5 was amplified and constructed onto the pGADT7 vector (the pGADT7 vector was digested with the *Eco*RI enzyme). *FgAox* promoter region sequence (first 2000 bp of ATG) was amplified and constructed into the $pHIS_2$ vector ($pHIS_2$ vector was cut by *EcoRV* enzyme). The pGADT7 and $pHIS_2$ constructs were separately cotransformed with these recombinant vectors into the yeast strain Y187. The transformants were identified using SD/-Leu/-Trp dropout plates with 50 mM 3-AT (a reagent that inhibits self-activation).[40] This experiment was conducted in triplicate.

2.12. Cellular Localization of *FgAox*, *FgAod*2, and *FgAod*5 in *F. graminearum*. To further determine the cellular localization of *FgAox*, *FgAod*2, and *FgAod*5, we generated GFP fusion constructs for each gene in *F. graminearum*. Confocal microscopy (Leica TCS SP8, Germany) was used to observe the fluorescence signal and determine the subcellular localization of these proteins.

2.13. Statistical Analysis. All data were analyzed using SPSS version 14.0 software (SPSS Inc. Chicago, IL). Fisher's LSD test (P = 0.05) was applied to obtain the standard errors and identify significant differences among the EC_{50} values of pyraclostrobin to different mutants, sensitive strains, and the relative expression levels of each gene assessed in this study. Each treatment was replicated three times, and the experiment was repeated three times.

3. RESULTS

3.1. *FgCox* Deletion Mutants Remarkably Decreased the Sensitivity to Pyraclostrobin. A schematic drawing of the generation of ΔFgCox is shown in Figure S1. The verification result of each *FgCox* gene deletion mutant using the Southern blot is shown in Figure S2. The primers used in this study are shown in Table S1.

Table 1. Sensitivity of *FgCox* Deletion Mutants in *F. graminearum* to Pyraclostrobin

strains	EC_{50} (μg/mL)	strains	EC_{50} (μg/mL)
PH-1	0.014 ± 0.0040 hi[a]	PH-1	0.014 ± 0.0040 hi
ΔFgCox4−2	1.42 ± 0.43 b	ΔFgCox10−9	0.73 ± 0.085 cdef
ΔFgCox4−3	1.30 ± 0.41 b	ΔFgCox10−10	0.85 ± 0.071 cde
ΔFgCox4-C	0.030 ± 0.0075 hi	ΔFgCox10-C	0.035 ± 0.022 hi
ΔFgCox5A-7	0.80 ± 0.16 cdef	ΔFgCox11−30	0.84 ± 0.051 bc
ΔFgCox5A-8	0.77 ± 0.21 cdef	ΔFgCox11−44	0.83 ± 0.049 bc
ΔFgCox5A-C	0.035 ± 0.019 hi	ΔFgCox11-C	0.038 ± 0.0090 hi
ΔFgCox5B-2	0.69 ± 0.20 defg	ΔFgCox15−22	0.64 ± 0.11 bcd
ΔFgCox5B-6	0.56 ± 0.035 cdef	ΔFgCox15−28	1.02 ± 0.15 bcd
ΔFgCox5B-C	0.085 ± 0.010 hi	ΔFgCox15-C	0.040 ± 0.015 hi
ΔFgCox6A-6	0.086 ± 0.038 hi	ΔFgCox16−7	0.48 ± 0.033 efghi
ΔFgCox6A-36	0.050 ± 0.020 hi	ΔFgCox16−15	0.64 ± 0.053 cdefg
ΔFgCox6A-C	0.017 ± 0.0018 hi	ΔFgCox16-C	0.022 ± 0.0017 hi
ΔFgCox6B-1	0.22 ± 0.027 ghi	ΔFgCox17−49	1.86 ± 0.57 a
ΔFgCox6B-4	0.22 ± 0.044 ghi	ΔFgCox17−75	2.58 ± 0.27 a
ΔFgCox6B-C	0.061 ± 0.0015 hi	ΔFgCox17-C	0.023 ± 0.012 hi
ΔFgCox7A-4	0.39 ± 0.046 fghi	ΔFgCox18−1	0.016 ± 0.006 hi
ΔFgCox7A-60	0.54 ± 0.026 efgh	ΔFgCox18−7	0.030 ± 0.011 hi
ΔFgCox7A-C	0.038 ± 0.0051 hi	ΔFgCox18-C	0.017 ± 0.015 hi
ΔFgCox7C-60	0.032 ± 0.0015 hi	ΔFgCox20−26	0.58 ± 0.14 efghi
ΔFgCox7C-90	0.050 ± 0.0035 hi	ΔFgCox20−39	0.77 ± 0.22 cde
ΔFgCox7C-C	0.017 ± 0.0023 hi	ΔFgCox20-C	0.019 ± 0.0061 hi

[a]The different letter means there are significant differences ($P < 0.05$).

Table 2. Sensitivity of *FgCox* Overexpression Mutants in *F. graminearum* to Pyraclostrobin

strains	OE[a]	EC_{50}[b] (μg/mL)	strains	OE	EC_{50} (μg/mL)
PH-1	1.05 ± 0.23	0.014 ± 0.0040 a[c]	PH-1	1.05 ± 0.23	0.014 ± 0.0040[a]
PH-1^{OECox4}-1	26.21 ± 9.36	0.0067 ± 0.0016 bcd	PH-1^{OECox10}-7	16.91 ± 1.84	0.0056 ± 0.0015 bcd
PH-1^{OECox4}-2	29.41 ± 2.48	0.0063 ± 0.0013 bcd	PH-1^{OECox10}-9	43.13 ± 7.39	0.0049 ± 0.00090 cd
PH-1^{OECox5A}-1	7.44 ± 0.59	0.0057 ± 0.0011 bcd	PH-1^{OECox11}-3	21.36 ± 7.63	0.0065 ± 0.0017 bcd
PH-1^{OECox5A}-9	6.06 ± 0.48	0.0061 ± 0.0014 bcd	PH-1^{OECox11}-6	13.78 ± 1.50	0.0075 ± 0.0016 bcd
PH-1^{OECox5B}-1	12.72 ± 0.23	0.0031 ± 0.0010 d	PH-1^{OECox15}-2	64.92 ± 15.54	0.0095 ± 0.0043 bcd
PH-1^{OECox5B}-2	20.41 ± 1.11	0.0035 ± 0.0011 d	PH-1^{OECox15}-3	9.35 ± 4.18	0.0076 ± 0.0021 bcd
PH-1^{OECox6A}-2	88.81 ± 26.85	0.012 ± 0.0055 abcd	PH-1^{OECox16}-1	79.68 ± 19.07	0.0049 ± 0.0011 cd
PH-1^{OECox6A}-3	72.36 ± 21.88	0.012 ± 0.0055 abcd	PH-1^{OECox16}-2	4.30 ± 0.47	0.0071 ± 0.0015 bcd
PH-1^{OECox6B}-2	15.61 ± 0.28	0.012 ± 0.0050 abcd	PH-1^{OECox17}-1	27.77 ± 6.65	0.0052 ± 0.0010 cd
PH-1^{OECox6B}-4	19.66 ± 4.08	0.012 ± 0.0049 abcd	PH-1^{OECox17}-2	75.62 ± 24.17	0.0057 ± 0.0013 cd
PH-1^{OECox7A}-2	216.96 ± 69.35	0.0064 ± 0.0015 bcd	PH-1^{OECox18}-2	3.12 ± 0.88	0.015 ± 0.0073 abc
PH-1^{OECox7A}-3	176.77 ± 56.50	0.0063 ± 0.0016 bcd	PH-1^{OECox18}-4	3.28 ± 0.51	0.015 ± 0.0070 abc
PH-1^{OECox7C}-3	24.13 ± 5.01	0.016 ± 0.0075 ab	PH-1^{OECox20}-2	14.11 ± 0.060	0.0040 ± 0.0011 cd
PH-1^{OECox7C}-5	11.50 ± 0.050	0.015 ± 0.0074 abc	PH-1^{OECox20}-3	16.01 ± 0.98	0.0048 ± 0.0014 cd

[a]OE means that the relative expression level of the gene in it is overexpression. [b]The EC_{50} values of pyraclostrobin to PH-1 and *FgCox* overexpression mutants. [c]The different letter means that there are significant differences ($P < 0.05$).

Compared to the sensitive strains (PH-1 and complemented strain), 11 *FgCox* deletion mutants (ΔFgCox4, ΔFgCox5A, ΔFgCox5B, ΔFgCox6B, ΔFgCox7A, ΔFgCox10, ΔFgCox11, ΔFgCox15, ΔFgCox16, ΔFgCox17, and ΔFgCox20) exhibited resistance to pyraclostrobin (Figure 1). The average EC_{50} values of pyraclostrobin for these mutants ranged from 0.22 to 2.58 μg/mL; for sensitive strains, they ranged from 0.014 to 0.085 μg/mL. The ΔFgCox17 deletion mutants exhibited the most notable decrease in sensitivity, with an average EC_{50} value of 2.22 μg/mL, followed by the ΔFgCox4 deletion mutants with an average EC_{50} value of 1.36 μg/mL, which increased by 158.57 and 97.14 times compared to the EC_{50} value of PH-1, respectively. The sensitivity of ΔFgCox6A, ΔFgCox7C, and ΔFgCox18 mutants to pyraclostrobin did not change significantly; the average EC_{50} values of pyraclostrobin for these mutants were 0.032−0.086 μg/mL (Table 1).

3.2. *FgCox* Overexpression Mutants Significantly Increased the Sensitivity to Pyraclostrobin. Compared to PH-1, 10 overexpression mutants of *FgCox* genes (*FgCox*4, *FgCox5A*, *FgCox5B*, *FgCox7A*, *FgCox*10, *FgCox*11, *FgCox*15, *FgCox*16, *FgCox*17, and *FgCox*20) exhibited hypersensitivity to pyraclostrobin. Among them, the *FgCox5B* overexpression mutants showed the highest sensitivity to pyraclostrobin, with an average EC_{50} value of 0.003 ± 0.0002 μg/mL, compared to 0.013 ± 0.0049 μg/mL for PH-1. There was no significant difference in sensitivity to pyraclostrobin between the overexpression mutants of *FgCox6A*, *FgCox6B*, *FgCox7C*, *FgCox*18, and PH-1 (Table 2). These results indicated that

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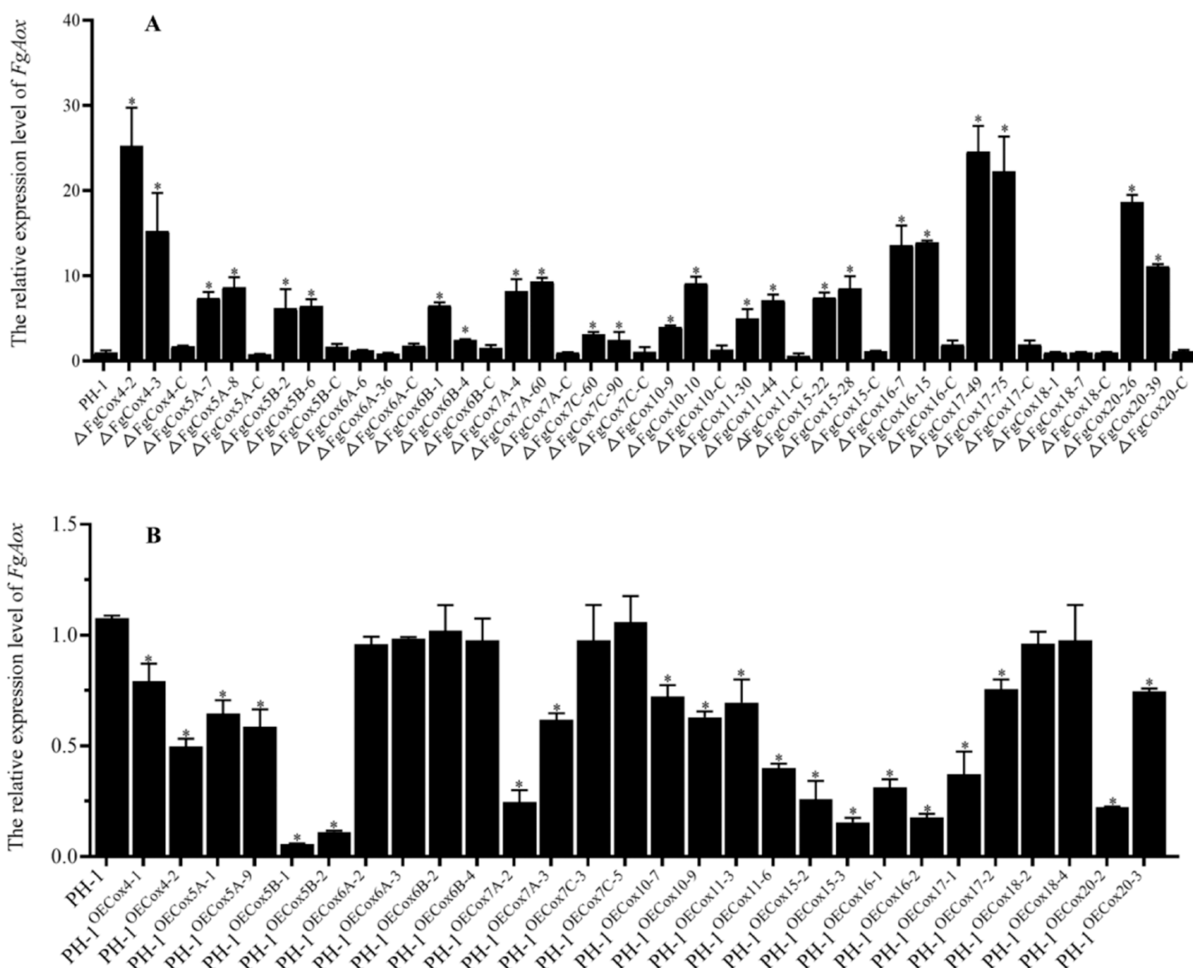

Figure 2. *FgCox* regulated the expression of *FgAox*. (A) Relative expression levels of *FgAox* in *FgCox* deletion mutants and complemented strain compared with PH-1 when treated with pyraclostrobin. (B) Relative expression levels of *FgAox* in *FgCox* overexpression mutants. * indicates significant differences ($P < 0.05$).

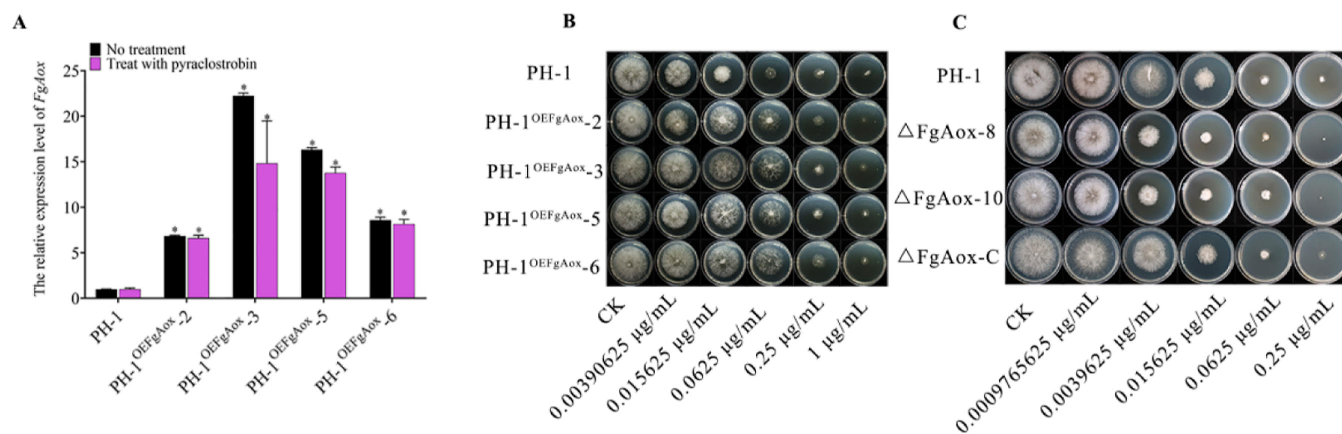

Figure 3. *FgAox* is involved in the sensitivity of *F. graminearum* to pyraclostrobin. (A) Relative expression levels of *FgAox* in *FgAox* overexpression mutants. * indicates significant differences ($P < 0.05$). (B) Sensitivity of *FgAox* overexpression mutants to pyraclostrobin. (C) Sensitivity of *FgAox* deletion mutants to pyraclostrobin.

FgCox genes are involved in the sensitivity of *F. graminearum* to pyraclostrobin.

3.3. *FgAox* Regulated the Sensitivity of *F. graminearum* to Pyraclostrobin. The RNA-Seq results revealed that the expression levels of *FgAox* in ΔFgCox4−2 and ΔFgCox17−75 mutants were increased by 3.93 and 4.21-fold, respectively, compared to PH-1 after treatment with pyraclostrobin (Figure S3). The relative expression levels of *FgAox* in all *FgCox* deletion mutants, except for ΔFgCox6A and ΔFgCox18, were significantly increased (Figure 2A). Among the deletion mutants, ΔFgCox4−2 showed the highest increase in the relative expression levels of *FgAox*, with a 25.25

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Table 3. Sensitivity of *FgAox* Overexpression Mutants to Pyraclostrobin

	no treatment[a]	pyraclostrobin[b]		
strains	*FgAox*[c]	EC_{50} (μg/mL)	RF[d]	*FgAox*
PH-1	1.00 ± 0.010 c[e]	0.014 ± 0.0040 c		1.00 ± 0.070 c
PH-1^{OEAox}-2	6.84 ± 0.050 b	0.11 ± 0.034 b	7.86	6.62 ± 0.18 b
PH-1^{OEAox}-3	22.26 ± 0.16 a	0.16 ± 0.054 a	11.43	14.84 ± 2.68 a
PH-1^{OEAox}-5	9.13 ± 0.030 b	0.14 ± 0.034 b	10.00	13.78 ± 0.37 a
PH-1^{OEAox}-6	8.59 ± 0.18 b	0.21 ± 0.044 a	15.00	8.15 ± 0.29 b

[a]No treatment means the relative expression of *FgAox* in *FgAox* overexpression without any treatment. [b]Pyraclostrobin means the strain treated with pyraclostrobin. [c]Means the relative expression levels of *FgAox*. [d]RF = EC_{50} value of overexpression mutants/EC_{50} value of PH-1. [e]The different letter means there are significant differences ($P < 0.05$).

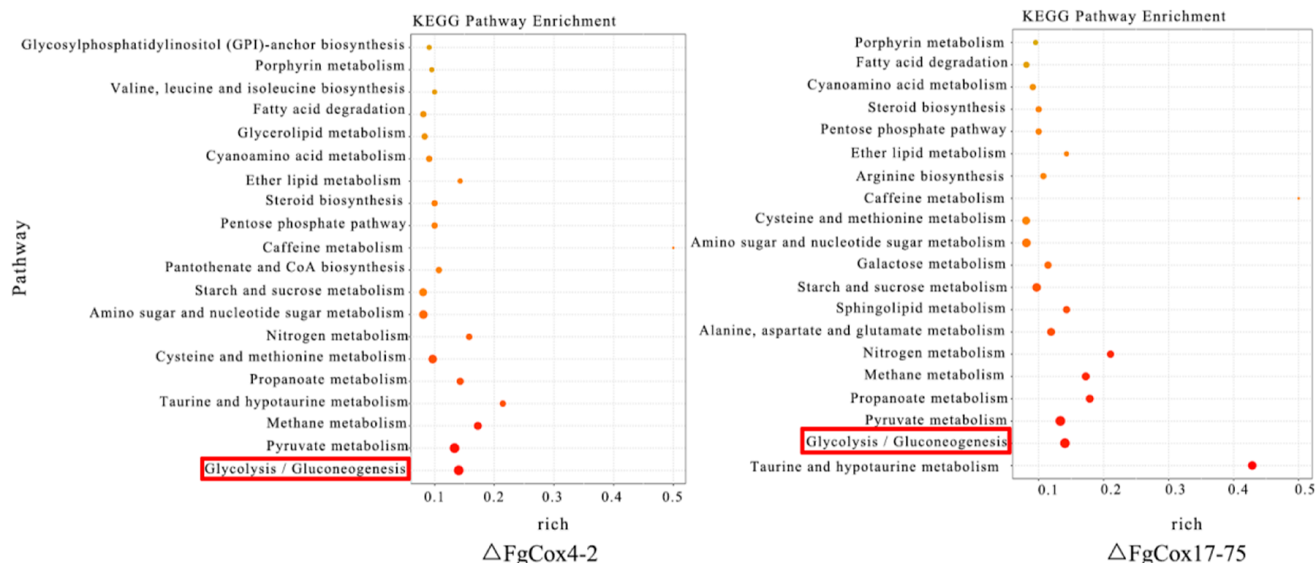

Figure 4. KEGG pathway enrichment assay in ΔFgCox4−2 and ΔFgCox17−75 mutants when treated with pyraclostrobin. Glycolysis/gluconeogenesis pathways marked with red box.

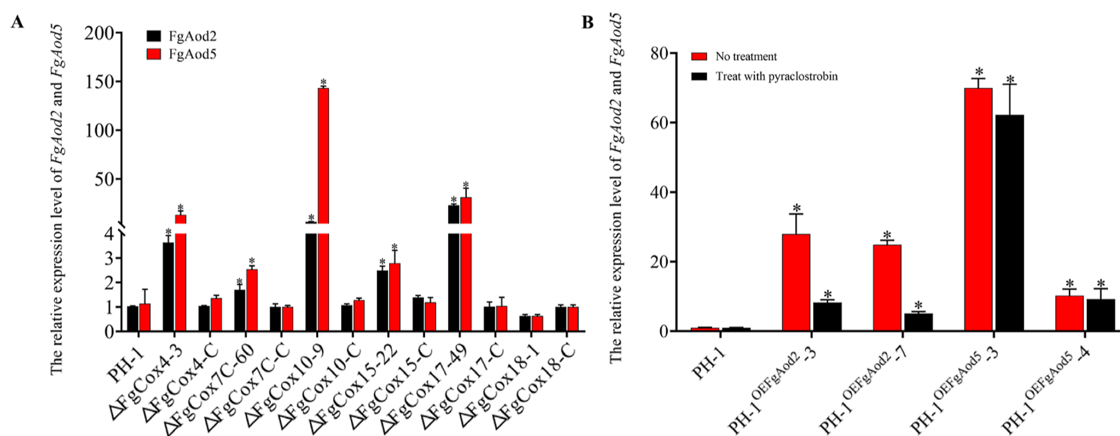

Figure 5. Relative expression levels of FgAod2 and FgAod5. (A) Relative expression levels of *FgAod2* and *FgAod5* in six Cox deletion mutants, their complemented strain, and PH-1. (B) Relative expression levels of *FgAod2* and *FgAod5* in *FgAod2* and *FgAod5* overexpression mutants, respectively. * indicates significant differences ($P < 0.05$)..

± 2.59-fold increase compared to that of PH-1 (Table S3). The relative expression levels of *FgAox* in all *FgCox* overexpression mutants, except *FgCox6A*, *FgCox6B*, *FgCox7C*, and *FgCox18* overexpression mutants, were significantly decreased (Figure 2B and Table S4). The result indicated that *FgCox* regulated the expression of *FgAox*.

The relative expression levels of *FgAox* in the *FgAox* overexpression mutants were significantly increased by 6.62−14.84 times when treated with pyraclostrobin (Figure 3A and Table 3). The sensitivity test results showed that *FgAox* overexpression mutants exhibited considerably decreased sensitivity to pyraclostrobin (Figure 3B). The EC_{50} of pyraclostrobin for the overexpression mutants or PH-1 was 0.11−0.21 and 0.014 ± 0.004 μg/mL, respectively (Table 3). These results indicated that overexpression of *FgAox* could

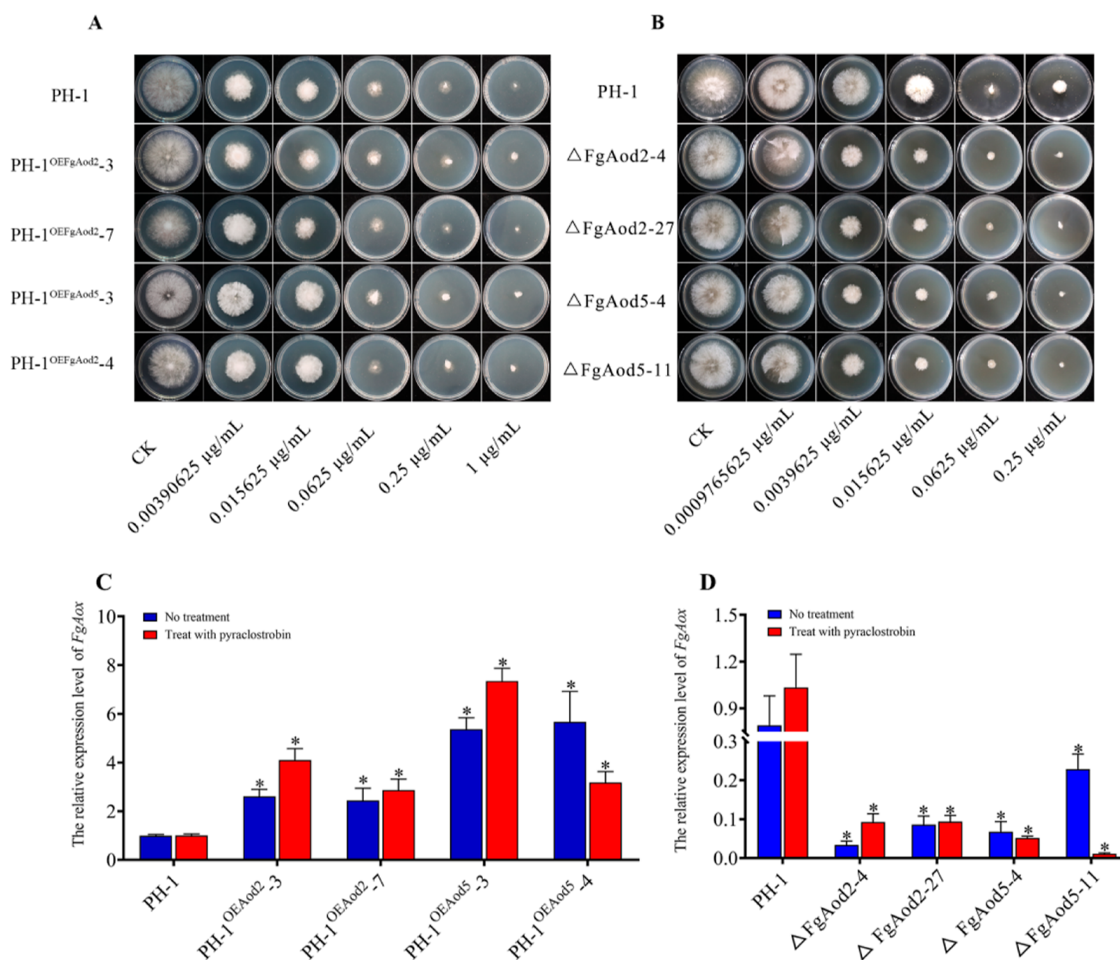

Figure 6. *FgAod*2 and *FgAod*5 involved in the sensitivity of *F. graminearum* to pyraclostrobin. (A) Sensitivity of *FgAod*2 and *FgAod*5 overexpression mutants to pyraclostrobin. (B) Sensitivity of *FgAod*2 and *FgAod*5 deletion mutants to pyraclostrobin. (C) Relative expression levels of *FgAox* in *FgAod*2 and *FgAod*5 overexpression mutants. (D) Relative expression levels of *FgAox* in ΔFgAod2 and ΔFgAod5 mutants. * indicates significant differences ($P < 0.05$).

significantly decrease the sensitivity of *F. graminearum* to pyraclostrobin.

The result of sensitivity tested in ΔFgAox mutants showed that the deletion mutants increased the prominent sensitivity to pyraclostrobin (Figure 3C). The verification of ΔFgAox is shown in Figure S4. When treated with pyraclostrobin, the EC_{50} of pyraclostrobin to ΔFgAox mutants, the complemented strain, and PH-1 was 0.0016 ± 0.0004, 0.010 ± 0.005, and 0.014 ± 0.004 μg/mL, respectively. The result indicated that deletion of *FgAox* could remarkably increase the sensitivity of *F. graminearum* to pyraclostrobin. In conclusion, *FgAox* could regulate the sensitivity of *F. graminearum* to pyraclostrobin.

3.4. *FgAod*2 and *FgAod*5 Regulated the Sensitivity of *F. graminearum* to Pyraclostrobin. RNA-seq results indicated that genes differentially expressed were enriched in the glycolysis/gluconeogenesis pathways in the ΔFgCox4−2 and ΔFgCox17−75 mutants (Figure 4). According to the KEGG annotation, *FgAod*2 and *FgAod*5 were identified as gluconeogenesis-related genes. The relative expression levels of *FgAod*2 and *FgAod*5 were significantly increased in five *FgCox* deletion mutants (ΔFgCox4, ΔFgCox7C, ΔFgCox10, ΔFgCox15, and ΔFgCox17), except for ΔFgCox18 mutants. The lowest upregulated expression of *FgAod*2 was 2.36 times in ΔFgCox10, and the highest upregulated expression was 6.48 times in ΔFgCox4−3. Similarly, the expression of *FgAod*5 showed a 4.00-fold increase in ΔFgCox7C with the lowest upregulation and a 193.09-fold increase in ΔFgCox10−9 with the highest upregulation (Figure 5A and Table S5).

We successfully generated overexpression mutants for *FgAod*2 and *FgAod*5, resulting in a 10.23−27.97-fold increase in their relative expression levels (Figure 5B). The sensitivity of *FgAod*2 and *FgAod*5 overexpression mutants to pyraclostrobin was significantly reduced compared to PH-1. The EC_{50} of pyraclostrobin to *FgAod*2 and *FgAod*5 overexpression mutants and PH-1 was 0.038 ± 0.0042, 0.067 ± 0.022, and 0.014 ± 0.0040 μg/mL, respectively (Figure 6A). Conversely, the sensitivity of the ΔFgAod2 and ΔFgAod5 mutants to pyraclostrobin was remarkably increased (Figure 6B). The EC_{50} of pyraclostrobin to ΔFgAod2, ΔFgAod5, and PH-1 was 0.0019 ± 0.00026, 0.0017 ± 0.00030, and 0.017 ± 0.0039 μg/mL, respectively. The result indicated that the *FgAod*2 and *FgAod*5 are involved in the sensitivity of *F. graminearum* to pyraclostrobin too.

3.5. *FgAox*, *FgAod*2, and *FgAod*5 Increased the Relative Expression Level in Pyraclostrobin-Resistant Mutants. In this study, we obtained four pyraclostrobin-resistant mutants (PH-1−1, PH-1−17, XX6−35−1, and XX6−35−10) from PH-1 and XX6−35 using fungicide taming and found that the resistance could inherit stability. The EC_{50} values of pyraclostrobin to wild-type strain and resistance

mutants are shown in Table 4. The relative expression level of *FgAox*, *FgAod2*, and *FgAod5* in pyraclostrobin-resistant samples

Table 4. Sensitivity of Pyraclostrobin-Resistant Mutants to Pyraclostrobin

strains	first[a] EC$_{50}$ (μg/mL)	first[a] RF[c]	10st[b] EC$_{50}$ (μg/mL)	10st[b] RF
PH-1	0.012 ± 0.0045		0.019 ± 0.0079	
PH-1−1	0.12 ± 0.0036	10	0.60 ± 0.14	31.42
PH-1−17	0.082 ± 0.013	6.83	0.60 ± 0.040	31.26
XX6−35	0.0066 ± 0.0030		0.031 ± 0.0058	
XX6−35−1	0.067 ± 0.017	10.21	0.48 ± 0.080	15.21
XX6−35−10	0.066 ± 0.032	9.98	0.37 ± 0.23	11.96

[a]First means the first generation of pyraclostrobin-resistant mutants on PDA plates. [b]10st means the pyraclostrobin-resistant mutants were transferred 10 times on PDA plates. [c]RF = EC$_{50}$ value of resistant mutants/ EC$_{50}$ value of parental strain.

was significantly increased compared with that in PH-1 and XX6−35 (Table S6). The results further indicated that *FgAox*, *FgAod2*, and *FgAod5* were involved in the sensitivity to pyraclostrobin in *F. graminearum*.

3.6. FgAod2 and FgAod5 Were the Transcription Factors of *FgAox* in *F. graminearum*. The relative expression level of *FgAox* in *FgAod2* and *FgAod5* overexpression mutants was significantly increased (Figure 6C and Table S7), while it was remarkably decreased in ΔFgAod2 and ΔFgAod5 mutants (Figure 6D and Table S7). The result indicated that *FgAod2* and *FgAod5* in *F. graminearum* have a significant impact on the expression of *FgAox*.

Through homologous comparison, we identified RDS2 as the homologous protein of FgAod2 and FgAod5 in *S. cerevisiae*. In the JASPAR database (https://jaspar.elixir.no/matrix/MA0362.1/), we found the binding motif of transcription factor RDS2, and this binding sequence (TCGG) was also present in the promoter region of *FgAox*. Furthermore, using Y1H analysis, we confirmed that FgAod2 and FgAod5 act as transcription factors for *FgAox* (Figure S5).

3.7. Cellular Localization of *FgAox*, *FgAod2*, and *FgAod5* in *F. graminearum*. The results indicated that *FgAox* was localized in the mitochondria, as confirmed by comparison with the mitochondria-specific dye Mito-Tracker.[41] Additionally, *FgAod2* and *FgAod5* were found to be located in the cell nucleus, as observed using DAPI staining (Figure 7).[42]

4. DISCUSSION

Cox, also referred to as mitochondrial respiratory chain complex IV, is a multisubunit complex situated in the inner mitochondrial membrane.[13,14] The structure of Cox has long been elucidated through techniques such as X-ray crystallography and electron microscopy. In 1995, Tsukihara and Iwata et al. reported the X-ray structures of Cox from bovine and bacterial sources, respectively.[42] In *S. cerevisiae*, Cox is composed of 24 subunits; in mammals, it consists of 23 subunits; and in *Arabidopsis thaliana*, it is made up of 14 subunits.[43] Despite extensive research on Cox in yeast, mammals, and plants, studies on Cox in plant pathogenic fungi remain largely unexplored. In this study, through homology comparison, 17 *FgCox* subunits were identified in *F. graminearum*, with Cox1, Cox2, and Cox3 encoded by mitochondrial genes, making it impossible to obtain deletion mutants for these genes in our lab. The remaining 14 subunits are encoded by nuclear genes. Therefore, we generated 14 *FgCox* gene deletion and overexpression mutants in *F. graminearum* to investigate their role in pyraclostrobin sensitivity. The majority of *FgCox* deletion mutants displayed resistance to the QoI fungicide pyraclostrobin (Figure 1 and Table 1), while *FgCox* overexpression mutants showed hypersensitivity, indicating *FgCox*'s crucial role in determining *F. graminearum*'s sensitivity to pyraclostrobin (Table 2).

Out of 14 *FgCox* gene deletion mutants, 11 exhibited resistance to pyraclostrobin, while 3 did not. Notably, two of these nonresponsive mutants (ΔFgCox6A and ΔFgCox18) exhibited normal growth (Figure 1), suggesting that these specific *FgCox* genes may not significantly impact the biological functions of *F. graminearum* and their deletion does not affect pyraclostrobin sensitivity. Additionally, the expression levels of *FgAox* in these three mutants did not significantly differ from those in wild-type strain PH-1 (Table S3). This lack of significant difference in *FgAox* expression is likely the main reason for their unchanged sensitivity to pyraclostrobin.

The relative expression levels of *FgAox* in most *FgCox* overexpression mutants, except for the *FgCox6A*, *FgCox6B*, *FgCox7C*, and *FgCox18* overexpression mutants, were significantly decreased (Figure 2B and Table S4). These results are consistent with the sensitivity of the overexpressed mutants to pyraclostrobin, further indicating that *FgCox* regulates the sensitivity of *F. graminearum* to pyraclostrobin by modulating the expression level of *FgAox*. Not all deletion mutants or overexpression mutants are involved in the sensitivity change to pyraclostrobin, which implies that not all *FgCox* genes are equally involved in the pathways targeted by the fungicide.

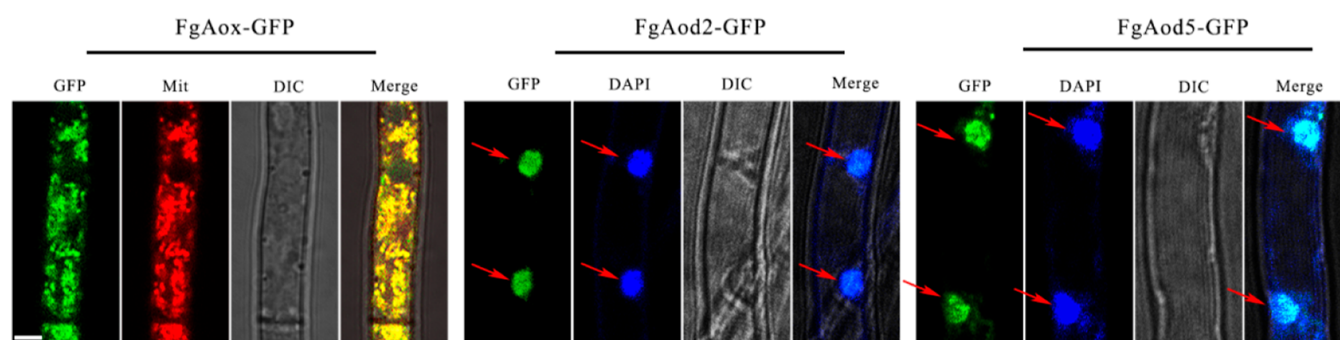

Figure 7. Cellular localization of *FgAox*, *FgAod2*, and *FgAod5* in *F. graminearum*. Bar = 10 μm.

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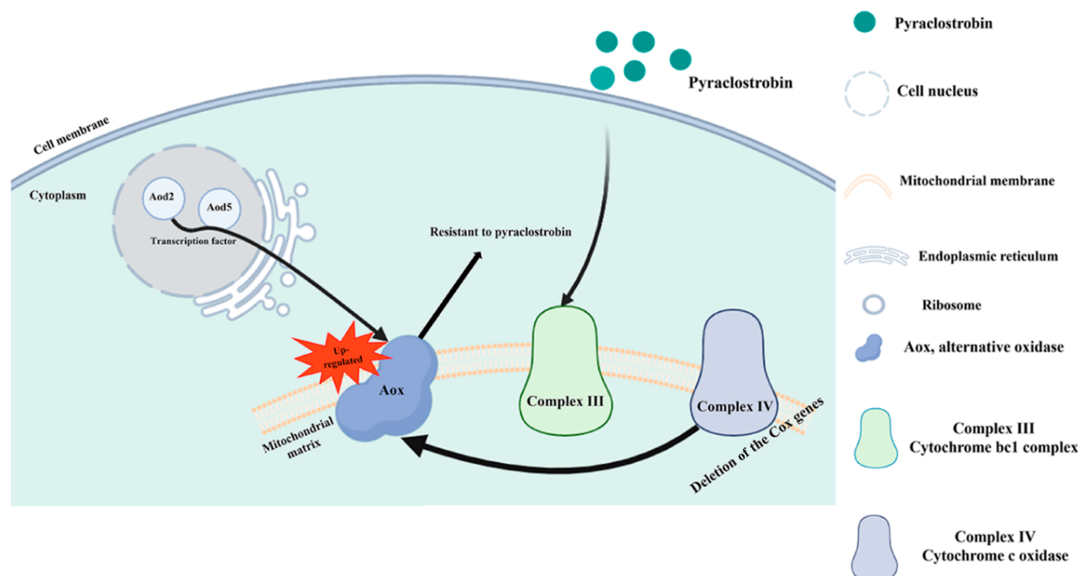

Figure 8. Proposed model suggests that pyraclostrobin sensitivity in *F. graminearum* is regulated by *FgCox*. Deletion of *FgCox* leads to an upregulation of expression levels for *FgAod2* and *FgAod5*, followed by an upregulation of *FgAox* expression. This cascade of gene expression changes ultimately reduces the sensitivity of *F. graminearum* to pyraclostrobin.

Some may have redundant functions that are compensated for by other genes.

Based on previous research, the resistance mechanisms to QoI fungicides can be categorized into two main types. First, there are point mutations in the target gene *Cytb*, such as the substitution of glycine at position 143 with alanine (G143A), which is associated with stronger resistance to these fungicides. A moderate level of resistance is conferred by other specific point mutations, such as the substitution of phenylalanine with leucine at position 129 (F129L) or the replacement of glycine with arginine at position 137 (G137R).[23] Second, the Aox pathway acts as a branch of the ETC. Aox is an enzyme found in the mitochondria of plants, fungi, and some other organisms.[25] It provides a bypass in the ETC, circumventing complexes III and IV. Aox transfers electrons directly from coenzyme Q to oxygen, forming water. Although this pathway does not generate ATP, it helps alleviate oxidative stress caused by interruptions in the ETC and maintains the flow of metabolites.[37,38] The upregulation of *Aox* could reduce the sensitivity of plant pathogenic fungi to QoI fungicide. For example, in *L. theobromae*, the expression of *Aox* was significantly upregulated by 33-fold, leading to notable resistance to pyraclostrobin.[24] Similarly, in *M. graminicola*, elevated *Aox* expression reduced the pathogen's sensitivity to pyraclostrobin.[25] In this study, RNA-Seq and RT-qPCR analysis revealed a significant upregulation of *FgAox* in Cox deletion mutants (Figure 3A and S3). We generated *FgAox* overexpression mutants in *F. graminearum*, which showed a marked decrease in pyraclostrobin sensitivity (Figure 3B). The EC_{50} of pyraclostrobin for overexpression mutants was 7.58−15.01 times higher than that for PH-1 (Table 3). Thus, the upregulated expression of *FgAox* in *FgCox* deletion mutants is responsible for their pyraclostrobin resistance. Conversely, *FgAox* deletion mutants exhibited increased sensitivity to the fungicide (Figure 3C), further confirming *FgAox*'s role in regulating pyraclostrobin sensitivity in *F. graminearum*.

FgAox regulation was explored through the transcription factors FgAod2 and FgAod5, homologous to Aod2 and Aod5 in *N. crassa*.[37,38] RNA-Seq analysis indicated significant changes in genes involved in glycolysis/gluconeogenesis pathways in ΔFgCox4 and ΔFgCox17 mutants treated with pyraclostrobin (Figure 4). This suggests a possible link between these pathways and the susceptibility of *FgCox* deletion mutants to pyraclostrobin. Overexpression and deletion mutants of FgAod2 and FgAod5 were constructed, revealing that overexpression decreased sensitivity to pyraclostrobin, while deletion increased it (Figure 6A,B). Relative expression assays showed significant upregulation of *FgAox* in FgAod2 and FgAod5 overexpression mutants and downregulation in their deletion mutants (Figure 6C,D). Y1H assays confirmed FgAod2 and FgAod5 as transcription factors for *FgAox* (Figure S5), demonstrating their role in pyraclostrobin sensitivity through *FgAox* regulation.

In conclusion, the *FgCox* genes are pivotal in modulating the sensitivity of *F. graminearum* to pyraclostrobin. Deletion of *FgCox* genes leads to upregulation of transcription factors FgAod2 and FgAod5, which in turn upregulate *FgAox* expression, reducing the fungus's sensitivity to pyraclostrobin (Figure 8). This study enhances our understanding of the mechanisms of resistance to QoI fungicides in *F. graminearum* and may guide the development of more effective fungicide management strategies.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jafc.4c04246.

Schematic drawings, verification, expression levels of *FgAox*, *FgAod2*, and *FgAod5*, Y1H assay, and primers used in this study (PDF)

AUTHOR INFORMATION

Corresponding Author

Yiping Hou − *College of Plant Protection, Nanjing Agricultural University, Nanjing, Jiangsu Province 210095, China*; Email: houyiping@njau.edu.cn

Authors

Zhiwen Wu − *College of Plant Protection, Nanjing Agricultural University, Nanjing, Jiangsu Province 210095, China;* orcid.org/0000-0002-2785-5581

Furong Chen − *College of Plant Protection, Nanjing Agricultural University, Nanjing, Jiangsu Province 210095, China*

Lingrong Zhang − *College of Plant Protection, Nanjing Agricultural University, Nanjing, Jiangsu Province 210095, China*

Mingguo Zhou − *College of Plant Protection, Nanjing Agricultural University, Nanjing, Jiangsu Province 210095, China*

Complete contact information is available at: https://pubs.acs.org/10.1021/acs.jafc.4c04246

Author Contributions

Z.W. conducted most of the experiments, collected the test data, and drafted the original article. M.Z., F.C., and L.Z. participated in the revision of the article. Y.H. designed the research and revised the manuscript.

Notes

The authors declare no competing financial interest.

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ABBREVIATIONS USED

Cox, cytochrome *c* oxidase; Aox, alternative oxidase; Aod, alternative oxidase deficiency; QoI, quinone outside inhibitor; EC_{50}, 50% effective concentration

REFERENCES

(1) Xin, L. Chemical fertilizer rate, use efficiency and reduction of cereal crops in China, 1998−2018. *J. Geogr. Sci.* **2022**, *32* (1), 65−78.

(2) Filip, E.; Woronko, K.; Stepien, E.; Czarniecka, N. An overview of factors affecting the functional quality of common wheat (*Triticum aestivum* L.). *Int. J. Mol. Sci.* **2023**, *24* (8), 7524.

(3) Faostat. Database of agricultural production, food and agriculture organization of the United Nations, Rome, 2020. http://faostat.fao.org/default.aspx (accessed March 20, 2024).

(4) Abdalla, A.; Stellmacher, T.; Becker, M. Trends and prospects of change in wheat self-sufficiency in Egypt. *Agriculture-Basel.* **2022**, *13* (1), 7.

(5) Mitura, K.; Cacak-Pietrzak, G.; Feledyn-Szewczyk, B.; Szablewski, T.; Studnicki, M. Yield and grain quality of common wheat (*Triticum aestivum* L.) depending on the different farming systems (organic vs. integrated vs. conventional). *Plants* **2023**, *12* (5), 1022.

(6) Alisaac, E.; Mahlein, A. Fusarium head blight on wheat: biology, modern detection and diagnosis and integrated disease management. *Toxins* **2023**, *15* (3), 192.

(7) Jevtic, R.; Skenderovic, N.; Zupunski, V.; Lalosevic, M.; Orbovic, B.; Masirevic, S. The relationship between Fusarium head blight traits, thousand-kernel weight, and yield in winter wheat. *Sci. Agric.* **2022**, *79*, No. e20200046.

(8) Jevtic, R.; Skenderovic, N.; Zupunski, V.; Lalosevic, M.; Orbovic, B.; Masirevic, S.; Bagi, F. Association between yield loss and Fusarium head blight traits in resistant and susceptible winter wheat cultivars. *J. Plant Dis. Prot.* **2021**, *128* (4), 1013−1022.

(9) Chen, Y.; Kistler, H.; Ma, Z. Fusarium graminearum trichothecene mycotoxins: biosynthesis, regulation, and management. *Annu. Rev. Phytopathol.* **2019**, *57* (1), 15−39.

(10) Ji, J.; Huang, H.; Li, L.; Ye, J.; Sun, J.; Sheng, L.; Ye, Y.; Zheng, Y.; Zhang, Z.; Sun, X. Volatile metabolite profiling of wheat kernels contaminated by *Fusarium graminearum*. *J. Agric. Food Chem.* **2023**, *71* (7), 3508−3517.

(11) Chen, L.; Yang, J.; Wang, H.; Yang, X.; Zhang, C.; Zhao, Z.; Wang, J. NX toxins: New threat posed by *Fusarium graminearum* species complex. *Trends Food Sci. Technol.* **2022**, *119*, 179−191.

(12) Johns, L.; Bebber, D.; Gurr, S.; Brown, N. Emerging health threat and cost of Fusarium mycotoxins in European wheat. *Nat. Food.* **2022**, *3* (12), 1014−1019.

(13) Yoshikawa, S.; Shimada, A. Reaction mechanism of cytochrome c oxidase. *Chem. Rev.* **2015**, *115*, 1936−1989.

(14) Wikström, M.; Krab, K.; Sharma, V. Oxygen activation and energy conservation by cytochrome c oxidase. *Chem. Rev.* **2018**, *118* (5), 2469−2490.

(15) Sarkar, A.; Chattopadhyay, S.; Mukherjee, M.; Ghosh Dey, S.; Dey, A. Assembly of redox active metallo-enzymes and metallo-peptides on electrodes: A biological constructs to probe natural processes. *Curr. Opin. Chem. Biol.* **2022**, *68*, 102142.

(16) Sato, W.; Ishimori, K. Regulation of electron transfer in the terminal step of the respiratory chain. *Biochem. Soc. Trans.* **2023**, *51* (4), 1611−1619.

(17) Adams, P.; Lightowlers, R.; Turnbull, D. Molecular analysis of cytochrome c oxidase deficiency in Leigh's syndrome. *Ann. Neurol.* **1997**, *41* (2), 268−270.

(18) Parker, W.; Mahr, N.; Filley, C.; Parks, J.; Hughes, D.; Young, D.; Cullum, C. Reduced platelet cytochrome-c-oxidase activity in alzheimers-disease. *Neurol.* **1994**, *44* (6), 1086.

(19) Schertl, P.; Braun, H. P. Respiratory electron transfer pathways in plant mitochondria. *Front. Plant Sci.* **2014**, *5*, 163.

(20) Ali, M.; Oliva, C.; Flor, S.; Goswami, P.; Griguer, C. Cytochrome c oxidase mediates labile iron level and radioresistance in glioblastoma. *Free Radic. Biol. Med.* **2022**, *185*, 25−35.

(21) Oliva, C.; Markert, T.; Ross, L.; White, E.; Rasmussen, L.; Zhang, W.; Everts, M.; Moellering, D.; Bailey, S.; Suto, M.; Griguer, C. Identification of small molecule inhibitors of human cytochrome c oxidase that target chemoresistant glioma cells. *J. Biol. Chem.* **2016**, *291* (46), 24188−24199.

(22) Yun, Y.; Guo, P.; Zhang, J.; You, H.; Guo, P.; Deng, H.; Hao, Y.; Zhang, L.; Wang, X.; Abubakar, Y.; Zhou, J.; Lu, G.; Wang, Z.; Zheng, W. Flippases play specific but distinct roles in the development, pathogenicity, and secondary metabolism of Fusarium graminearum. *Mol. Plant Pathol.* **2020**, *21* (10), 1307−1321.

(23) Sierotzki, H.; Wullschleger, J.; Gisi, U. Point mutation in cytochrome b gene conferring resistance to strobilurin fungicides in *Erysiphe graminis* f.sp.tritici field isolates. *Pestic. Biochem. Physiol.* **2000**, *68* (2), 107−112.

(24) Dong, G. R.; Zhang, Y.; Liang, X. Y.; Wang, M.; Ye, Q. Q.; Xian, X. W.; Yang, Y. Resistance characterization of the natural population and resistance mechanism to pyraclostrobin in *Lasiodiplodia theobromae*. *Pestic. Biochem. Physiol.* **2022**, *188*, 105232.

(25) Miguez, M.; Reeve, C.; Wood, P.; Hollomon, D. Alternative oxidase reduces the sensitivity of *Mycosphaerella graminicola* to QoI fungicide. *Pest Manag. Sci.* **2004**, *60* (1), 3−7.

(26) Mao, X.; Wu, Z.; Bi, C.; Wang, J.; Zhao, F.; Gao, J.; Hou, Y.; Zhou, M. Molecular and biochemical characterization of pydiflumetofen-resistant mutants of *Didymella bryoniae*. *J. Agric. Food Chem.* **2020**, *68* (34), 9120−9130.

(27) Osorio, J. M.; Adaskaveg, J. E.; Ogawa, J. M. Inhibition of mycelial growth of monilinia species and suppression and control of brown-rot blossom blight of almond with iprodione and e-0858. *Plant Dis.* **1994**, *78* (7), 712−716.

(28) Yu, J.; Hamari, Z.; Han, K.; Seo, J.; Reyes-Dominguez, Y.; Scazzocchio, C. Double-joint PCR: a PCR-based molecular tool for gene manipulations in filamentous fungi. *Fungal Genet. Biol.* **2004**, *41* (11), 973−981.

(29) Ge, C.; Duan, Y.; Zhou, M.; Chen, C. A protoplast transformation system for gene deletion and complementation in *Sclerotinia sclerotiorum*. *J. Phytopathol.* **2013**, *161* (11−12), 800−806.

(30) Liu, S.; Duan, Y.; Ge, C.; Chen, C.; Zhou, M. Functional analysis of the beta2-tubulin gene of *Fusarium graminearum* and the beta-tubulin gene of *Botrytis cinerea* by homologous replacement. *Pest Manag. Sci.* **2013**, *69* (5), 582−588.

(31) Hou, Y.; Chen, Y.; Wang, L.; Zheng, Y.; Liu, S.; Xu, J. Impact of pyraclostrobin on the growth of *Fusarium pseudograminearum* and its control efficacy against wheat crown rot in Henan Province, China. *J. Plant Pathol.* **2023**, *105* (4), 1499−1510.

(32) Li, L.; Ding, S.; Sharon, A.; Orbach, M.; Xu, J. Mir1 is highly upregulated and localized to nuclei during infectious hyphal growth in the rice blast fungus. *Mol. Plant-Microbe Interact.* **2007**, *20* (4), 448−458.

(33) Bourett, T.; Sweigard, J.; Czymmek, K.; Carroll, A.; Howard, R. Reef coral fluorescent proteins for visualizing fungal patho-gens. *Fungal Genet. Biol.* **2002**, *37* (3), 211−220.

(34) Heid, C.; Stevens, J.; Livak, K.; Williams, P. Real time quantitative PCR. *Genome Res.* **1996**, *6* (10), 986−994.

(35) Kimura, S.; Shibata, Y.; Oi, T.; Kawakita, K.; Takemoto, D. Effect of flutianil on the morphology and gene expression of powdery mildew. *J. Pestic. Sci.* **2021**, *46* (2), 206−213.

(36) Batra, R.; Huang, C.; Pan, Q. Validation of quantitative real time PCR for JAK2 mutation detection by using $2^{-\Delta\Delta ct}$ Method. *Lab. Invest.* **2011**, *91*, 448A.

(37) Descheneau, A.; Cleary, I.; Nargang, F. Genetic evidence for a regulatory pathway controlling alternative oxidase production in *Neurospora crassa*. *Genetics* **2005**, *169* (1), 123−135.

(38) Qi, Z.; Nargang, F. AOD2 and AOD5 localize to the nucleus and synergistically induce alternative oxidase in *Neurospora crassa*. *Mol. Biol. Cell* **2011**, *22*, 257.

(39) Kim, S.; Kim, C. Y.; Park, S. Y.; Kim, K. T.; Jeon, J.; Chung, H.; Choi, G.; Kwon, S.; Choi, J.; Jeon, J.; Jeon, J. S.; Khang, C. H.; Kang, S.; Lee, Y. H. Two nuclear effectors of the rice blast fungus modulate host immunity via transcriptional reprogramming. *Nat. Commun.* **2020**, *11* (1), 5845.

(40) Zhang, M.; Zhao, R.; Huang, K.; Huang, S.; Wang, H.; Wei, Z.; Li, Z.; Bian, M.; Jiang, W.; Wu, T.; Du, X. The OsWRKY63-OsWRKY76-OsDREB1B module regulates chilling tolerance in rice. *Plant J.* **2022**, *112* (2), 383−398.

(41) Wang, J.; Zhang, L.; Gao, L.; Chen, J.; Zhou, T.; Liu, Y.; Jiang, F. A bright, red-emitting water-soluble BODIPY fluorophore as an alternative to the commercial Mito Tracker Red for high-resolution mitochondrial imaging. *J. Mater. Chem. B* **2021**, *9* (41), 8639−8645.

(42) Tarnowski, B.; Spinale, F.; Nicholson, J. DAPI as a useful stain for nuclear quantitation. *Biotechnol. Histochem.* **1991**, *66* (6), 296−302.

(43) Mansilla, N.; Racca, S.; Gras, D. E.; Gonzalez, D. H.; Welchen, E. The complexity of mitochondrial complex IV: An update of cytochrome c oxidase biogenesis in plants. *Int. J. Mol. Sci.* **2018**, *19* (3), 662.

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