https://doi.org/10.17221/52/2023-PPS

Identification and functional analysis of *glyoxal oxidase* gene from rubber tree anthracnose

Xuemei Xian[1,2], Kunmeng Li[3], Hui Liu[2], Zhenhui Wang[2], Kun Yuan[2], Yiyu Hu[2], Chengtian Feng[2], Meng Wang[1], Chunhua Lin[1], Qiguang He[2], Yu Zhang[1]**

[1]*Sanya Nanfan Research Institute of Hainan University, College of Plant Protection, Hainan University, Sanya and Haikou, China*

[2]*Hainan Key Laboratory for Cultivation and Physiology, Key Laboratory of Biology and Genetic Resources of Rubber Tree, Ministry of Agriculture and Rural Affairs, Rubber Research Institute, Chinese Academy of Tropical Agricultural Sciences, Haikou, China*

[3]*College of Tropical Crops, Yunnan Agricultural University, Puer, China*

**Corresponding authors: hqg11300817@163.com; yuzhang_rain@163.com*

Citation: Xian X.M., Li K.M., Liu H., Wang Z.H., Yuan K., Hu Y.Y., Feng C.T., Wang M., Lin C.H., He Q.G., Zhang Y. (2023): Identification and functional analysis of *glyoxal oxidase* gene from rubber tree anthracnose. Plant Protect. Sci., 59: 337–347.

Abstract: Glyoxal oxidase (GLOX, EC 1.2.3.15) is a class of hydrogen peroxide synthases involved in lignin degradation. Several *GLOX* genes of biotrophic and necrotrophic phytopathogenic fungi have been confirmed to contribute to fungal growth, development, and pathogenicity. However, the composition, sequence characteristics and functions of genes in the *GLOX* family of hemi-biotrophic phytopathogenic fungi remain poorly understood. This study identified a GLOX--encoding gene *CsGLOX* in the hemi-biotrophic phytopathogenic fungus *Colletotrichum siamense* by bioinformatics analysis and PCR cloning. The encoded protein contains a signal peptide and five cell wall stress-responsive component (WSC) domains at the N terminus, followed by a catalytic structural domain (AA5_1). *CsGLOX*-deficient mutants were constructed by homologous replacement, which significantly increased the formation of conidia and reduced the pathogenicity and the high-osmotic stress tolerance of the fungus. However, the deletions did not influence fungal filamentous growth. This study proposes that the *CsGLOX* gene is involved in pathogen conidial formation, pathogenicity, and osmotic stress response, which deepens our understanding of the pathogenic mechanism of hemi-biotrophic phytopathogenic fungus *C. siamense*.

Keywords: glyoxal oxidase; *Colletotrichum siamense*; pathogenicity; stress responses; pathogenic mechanism

Hevea brasiliensis Muell is the primary source of natural rubber production. Rubber tree anthracnose caused by hemi-biotrophic phytopathogenic fungi *Colletotrichum* species is among the most serious leaf diseases affecting rubber tree plantations and limiting cultivation (Liu et al. 2018; Song et al. 2022). This disease frequently occurs in leafing in early spring, responsible for the repeated defoliation of rubber trees and causing huge economic losses (Liu et al. 2018). The *C. gloeosporioides* species complexes and *C. acutatum* species complexes are causal agents of rubber tree anthracnose on rubber trees worldwide.

Supported by the Hainan Provincial Natural Science Foundation of China (No. 321QN331), the Central Public-interest Scientific Institution Basal Research Fund for Chinese Academy of Tropical Agricultural Sciences (No. 1630022022012) and the Earmarked Fund for China Agriculture Research System (CARS-33).

https://doi.org/10.17221/52/2023-PPS

C. siamense, which belongs to the *C. gloeosporioides* species complex, is China's main causal agent of rubber tree anthracnose (Cao et al. 2017; Liu et al. 2018). However, studies on its pathogenic mechanism are still relatively sparse, seriously restricting the research and development of relevant prevention and control strategies. Recently, the genome of *C. siamense* has been sequenced (Liu et al. 2020) and provides a good basis for a better understanding its molecular pathogenic mechanism.

Glyoxal oxidase (*GLOX*), which is widely found in eukaryotes, was first identified in the wood-rot fungus *Phanerodontia chrysosporium* (Kersten & Kirk 1987). It is a carbohydrate-active enzyme belonging to the "auxiliary activities" subfamily AA5_1 (Koschorreck et al. 2022; Fong & Brumer 2023) and catalyze the oxidation of aldehydes into carboxylic acids while reducing dioxygen to hydrogen peroxide (H_2O_2) (Kersten & Cullen 2014; Daou & Faulds 2017; Koschorreck et al. 2022; Fong & Brumer 2023). It was further reported that GLOXs from wood-rot fungi participate in the degradation of lignin by providing H_2O_2 for the oxidation reaction catalyzed by extracellular peroxidase (including lignin peroxidase, manganese peroxidase, and other multifunctional peroxidases) (Kersten et al. 1995; Bak et al. 2009; Takano et al. 2010; MacDonald et al. 2011; Kersten and Cullen 2014; Matityahu et al. 2015; Kadowaki et al. 2018; Wohlschlager et al. 2021). Only a few *GLOX* genes in phytopathogenic fungi have been identified and functionally verified to be involved in the regulation of filamentous growth and pathogenic development; however, its molecular mechanism is still largely unknown (Kan et al. 2004; Leuthner et al. 2005; Song et al. 2016). Other studies have also revealed the presence of GLOXs in phytopathogenic fungi by bioinformatics analysis of the genome or secretome and speculated that GLOXs from phytopathogenic fungi may belong to extracellular proteins and participate in pathogen-plant interactions (Meinhardt et al. 2014; González-Fernández et al. 2015; Vela-Corcía et al. 2016; Lopez et al. 2018; Haile et al. 2020; Ribeaucourt et al. 2021; Chandrasekar et al. 2022). Nonetheless, the relevant biological functions have not been demonstrated.

Thus far, the composition, sequence characteristics and functions of *GLOX* gene family in hemibiotrophic fungi have not been reported. In this study, a GLOX-encoding gene, named *CsGLOX*, was identified in the hemibiotrophic fungus *C. siamense* and characterized by the presence of signalP and five wall stress-responsive component (WSC) structural domains at the N-terminus, followed by an AA5_1 domain. Furthermore, *CsGLOX*-deficient mutants (Δ*CsGLOX*) were constructed by homologous replacement. To investigate the function of *CsGLOX*, the comparison of filamentous growth, conidial formation, pathogenicity, and stress response ability between *CsGLOX*-deficient mutants and wild-type strains was carried out.

MATERIAL AND METHODS

Fungal strains and culture conditions. The *C. siamens*e HN08 strain, isolated from diseased leaves of rubber trees in Qiongzhong Xinjing Farm, Hainan Province. Fungal cultures were grown at 28 ℃ on potato dextrose agar (PDA: 200 g/L potato, 20 g/L glucose, and 18 g/L agar) plates. *Escherichia coli* DH5a was cultured in Luria-Bertani (LB) medium supplemented with ampicillin antibiotics. The rubber tree cultivar, Reyan 7-33-97, was grown in a greenhouse at 28 ℃ with 16 h photoperiods and 55 % humidity.

Bioinformatics analyses. The protein sequences of *GLOXs* were available from the NCBI database (https://www.ncbi.nlm.nih.gov/) and taken as queries to search against the *C. siamense* (Liu et al. 2020) using reciprocal BLAST search programs. Protein domain prediction was performed using Smart (http://smart.embl-heidelberg.de/). Sequence alignments were generated using ClustalX (version 2.1). Figures displaying multiple sequence alignments of CsGLOX with the well-studied GLOXs (Table 1) were prepared by the ESPript software (version 3) (http://espript.ibcp.fr/ESPript/cgi-bin/ESPript.cgi) (Robert & Gouet 2014). A phylogenetic tree based on the maximum likelihood method with 1 000 bootstrap replicates was constructed using the CLUSTALW network program.

Gene clone and gene knockout plasmid construction. Specific primers CGF1/CGR1 (Table 2)

Table 1. List of query amino acid sequences of known *GLOXs* used for reciprocal BLAST

Accession number	Species	Protein name
XP_011388703.1	*Ustilago maydis*	UmGLOX1
XP_011325260.1	*Fusarium graminearum*	FgGLOX
CAD89674.1	*Botrytis cinerea*	BcGLOX
EHK15719	*Trichoderma virens*	TvGLOX

https://doi.org/10.17221/52/2023-PPS

Table 2. Primers used in the test

Primer	5'-3' sequence	Description/application
CGF1	ATGAGGGCCTCACAGTTGACATTCGTT	for *CsGLOX* gene clone
CGR1	TTACACGCCGGGGCGGGTG	
CGF2	gtcgacggtatcgataagcttGATGTGACCCAGCACCTCG	for insertion of the upstream sequence of the ORF region into vector pCX62-S
CGR2	ccggaattcgatatcaagcttGGTAACGAGTGTGTGCCAAGAGG	
CGF3	gcatgctctcaccgcggatccACCCGTCATGACACCGGG	for insertion of the downstream sequence of the ORF region into vector pCX62-S
CGR3	cgctctagaactagtggatccTGCAGTTTGTCGTCTATTGGGAT	
a	ATCGATATACTCGCCACCAACATAT	for validation of *CsGLOX* gene deletion mutant strains
b	ATGAGGGCCTCACAGTTGACA	
c	GTCAGTAAGTCGCTCTGGTTCAACA	
c1	GGTAACAACCTGTCCTGAGAATACA	
d	CCTCGACCCACGGTAACTCCA	
d1	GGGAGGAGAGATTTTCCACGA	
e	GTTCTGGCTCCCGATCTGAAC	
f	TTACACGCCGGGGCGGGT	
g	ACGTAGATGCTGGATGGGGTT	

were designed based on the candidate *CsGLOX* gene sequence, and the *CsGLOX* gene fragment was amplified using the genomic DNA and cDNA as templates, respectively. After PCR cloning, the *CsGLOX* gene fragments were cloned into the pMD18-T vector (TaKaRa, Japan) and sequenced at Huada Technology Company (China).

According to the ORF region sequence of *CsGLOX* obtained above, fragments with lengths of 1500 bp upstream and 1500 bp downstream of the ORF region were selected and used for designing the specific primers (upstream: CGF2/CGR2 and downstream: CGF3/CGR3) (Table 2). Subsequently, the upstream and downstream sequences were respectively ligated to the N-terminus and C-terminus of the sulfonylurea-resistant gene (*ILV1*) in a pCX62-S vector by homologous recombination (Song et al. 2022).

Construction of *CsGLOX* gene deletion mutant strains. In this study, homologous recombination was used for *CsGLOX* gene deletion. The schematic diagram is shown in Figure 3A. The *C. siamense* protoplasts used for target gene deletion were prepared and transformed by the methods of Lin et al. (2018). The transformants were screened on DCM medium (1.7 g/L yeast nitrogen base without amino acids, 2 g/L L-Asparagine monohydrate, 1 g/L NH_4NO_3, 10 g/L glucose, and 10 g/L agar) with 100 μg/mL sulfonylurea (Lin et al. 2018). Then, genomic DNA was obtained, and PCR verified *CsGLOX* gene deletion mutant strains with the specific primers (Table 2).

Phenotypic analysis of mutants. Cultures of selected transformants were compared to that of the WT strain for colony filamentous growth, conidial production, conidial and appressorial morphology, pathogenicity, and stress tolerance. Fresh conidia of WT and Δ*CsGLOX* strains were prepared as described in Song et al. (2022), and the concentration of spore suspension was adjusted to 1×10^6 /mL by a hemocytometer. For colony filamentous growth measurements, 2 μL fresh conidia were inoculated in the center of PDA (potato dextrose agar: 200 g/L potato, 20 g/L glucose and 20 g/L agar), CM (complete medium: 6 g/L yeast extract, 6 g/L tryptone, 10 g/L sucrose and 18 g/L agar) and MM agar medium (minimal medium: 7 g/L K_2HPO_4, 3 g/L KH_2PO_4, 1 g/L $(NH_4)SO_4$, 0.1 g/L $MgSO_4$, 0.5 g/L citrate sodium, 5 g/L glucose and 18 g/L agar), and filamentous growth diameters were measurements and photographed after culture at 28 ℃ for 3 days. Each treatment contained three technical replicates, and each experiment was repeated three times.

To determine conidial production, 2 μL fresh conidia were inoculated in the centre of PDA and cultured at 28 ℃ for six days, as described in Song et al. (2022). Subsequently, the conidia were washed with 1 mL sterile ddH_2O, and a hemocytometer counted the number of conidia. Each treatment contained eight independent experiments. In addition, the conidial morphology was observed and photographed using a confocal microscope (ZEISS LSM800, Germany) with a 40 × objective lens (ZEISS, Germany). A volume of 2 μL fresh conidia was cultured at 28 ℃ for 8 hours onto paraffin-coated slide glass and the appressorial morphology was observed and photographed.

The virulence of the WT and Δ*CsGLOX* strains was performed using droplet inoculations of 10 μL fresh conidia onto detached young rubber tree leaves

https://doi.org/10.17221/52/2023-PPS

wounded with needles. The inoculated tissues were incubated at 28 ℃ for 2 days under appropriate humidity and then photographed. The lesion area was calculated using ImageJ software. Each treatment contained ten independent replicates.

For the stress tolerance assay, NaCl (0.6 mol/L), sorbitol (1 mol/L), H_2O_2 (2 mmol/L), and congo red (6 μg/mL) were separately incorporated into PDA medium. Untreated PDA plates were used as controls. A volume of 2 μL fresh conidia was inoculated in the centre and the colony diameters were measured and photographed after four days of culture. Each treatment contained three technical replicates, and each experiment was repeated three times. The inhibition rate (%) was calculated as [Colony diameter (Control-Tested)] / Colony diameter (Control) × 100.

RESULTS

CsGLOX is a putative glyoxal oxidase. *GLOX* genes in biotrophic and necrotrophic phytopathogenic fungi have been functionally verified to be involved in the regulation of filamentous growth and pathogenic development (Kan et al. 2004; Leuthner et al. 2005; Song et al. 2016); however, the sequence characteristics and functions of the *GLOX* gene family of hemi-biotrophic phytopathogenic fungi have not been reported. Therefore, we conducted reciprocal BLAST searches using seven well-characterized GLOXs from NCBI databases (Table 1) to search for GLOX-encoding gene from the hemi-biotrophic phytopathogenic fungus *C. siamense* genome data and found a 3744-bp coding sequence (CDS) predicted to encode a GLOX ortholog, designated as *CsGLOX*. The CDS sequence of *CsGLOX* was confirmed by PCR (Table 2 and Figure 1A) using cDNA extracted from *C. siamense* as the template. The genomic clone of *CsGLOX* was also sequenced (Figure 1A) and deposited in GenBank under accession number OQ747757. Aligning the genomic sequence with the CDS showed that *CsGLOX* contained three introns of 49 bp, 51 bp, and 53 bp. The *CsGLOX* encoded a predicted polypeptide of 1247 amino acids with a molecular weight of 128.8 kDa. Protein domain prediction revealed the presence of SignalP at the N-terminus and five WSC domains, followed by one catalytic structural domain (AA5_1 domain) (Figure 1B). Aligning against the well-characterized fungal *GLOX* amino acid sequences from *Ustilago maydis* (UmGLOX1), *Fusarium graminearum* (FgGLOX), *Botrytis cinerea* (BcGLOX), and *Trichoderma virens* (TvGLOX) (Table 1) revealed that CsGLOX has conserved residues shared by the glyoxal oxidase family (including the copper coordinating active site and the radical redox site: Cys228, Tyr272, Tyr495, His496, and His581) (Figure 1C). These results indicated that CsGLOX is a copper radical oxidase.

Two main types of copper radical oxidases exist: glyoxal oxidases and galactose oxidases (EC 1.1.3.9) (Yin et al. 2015). Therefore, a phylogenetic tree based on the alignment of CsGLOX to glyoxal oxidase and galactose oxidase sequences was performed to predict whether CsGLOX is a glyoxal or galactose oxidase. The analysis revealed CsGLOX clusters with the other glyoxal oxidase proteins (Figure 2). This result, coupled with the sequence alignment and protein domain analysis, indicated that the CsGLOX is a glyoxal oxidase.

Construction of *CsGLOX* gene knockout mutants. To investigate the biological function of *CsGLOX* in *C. siamense*, the *CsGLOX* knockout mutant Δ*CsGLOX* was constructed. This was achieved by the targeted replacement of *CsGLOX* with the *ILV1* gene using the homologous recombination method (Figure 3A). In total, 33 single colonies were able to grow on sulfonylurea-containing DCM medium by PEG-mediated protoplast transformation technology. All single colonies obtained were further verified by PCR (Table 2 and Figure 3A) and two *CsGLOX* gene deletion mutants (named D21 and D28) were identified (Figure 3A). When wild-type strain HN08 (WT) genomic DNA was used as a template, it could be amplified by primers a/c (lane 1: 1779 bp), d/g (lane 2: 2152 bp), b/f (lane 5: *CsGLOX* gene 3897 bp) and e/f (lane 6: the internal sequence of *CsGLOX* gene 1383 bp) in the correct bank sizes, but could not be amplified by primers a/c1 (lane 3: 2781 bp) and d1/g (lane 4: 3935 bp) (Figure 3A). In contrast, the results of PCR using the DNA of the fungal transformants as a template were exactly opposite to those of the wild-type, which indicated that the *CsGLOX* gene was replaced by the *ILV1* gene (Figure 3A). Then, the PCR products from Δ*CsGLOX* (6336 bp) and WT (7407 bp) genomic DNA amplified by primers a/g were sequenced, which revealed that the ILV1 gene completely replaced the open reading frame of the *CsGLOX* gene. Therefore, D21 and D28 were indeed *CsGLOX*-deficient mutants.

***CsGLOX* gene is not essential for filamentous growth but negatively regulates the conidial**

https://doi.org/10.17221/52/2023-PPS

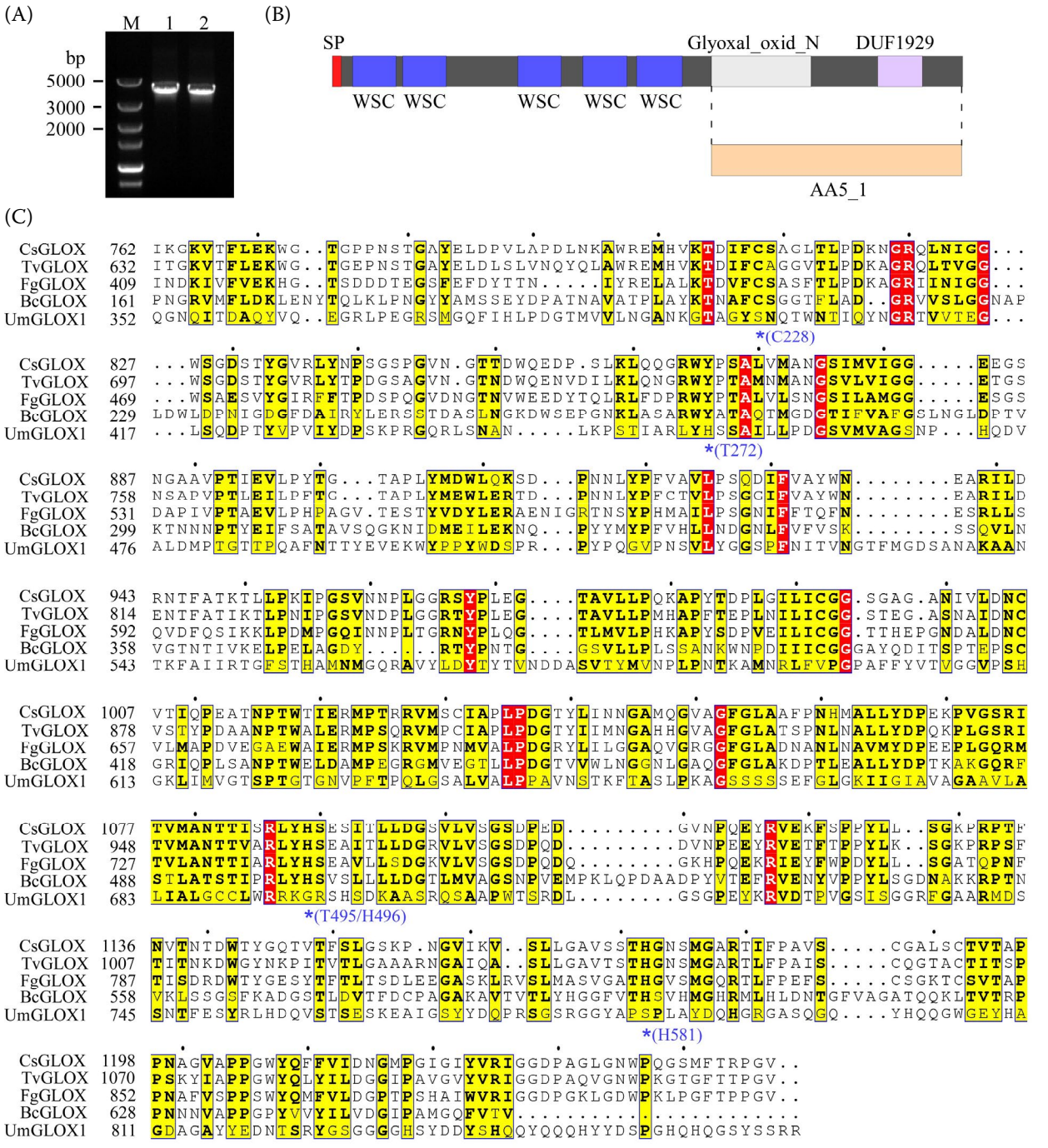

Figure 1. Cloning and sequence analysis of *CsGLOX* Gene

A – agarose gel electrophoresis of PCR products of the *CsGLOX* gene; M – DL5000 DNA marker; 1 – PCR products using DNA as template; 2 – PCR products using cDNA as template

B – the protein domains of CsGLOX protein

C – multiple alignment of C-terminal amino acid sequences between CsGLOX and previously reported fungi GLOXs; red – highly conserved amino acid residues; yellow – moderately conserved amino acid residues; blue asterisks – the putative active site residues

sporulation of *C. siamense*. It has been previously determined that *GLOX* deficiency results in severe defects in the hyphal development of *U. maydis* (Leuthner et al. 2005) and *T. virens*

https://doi.org/10.17221/52/2023-PPS

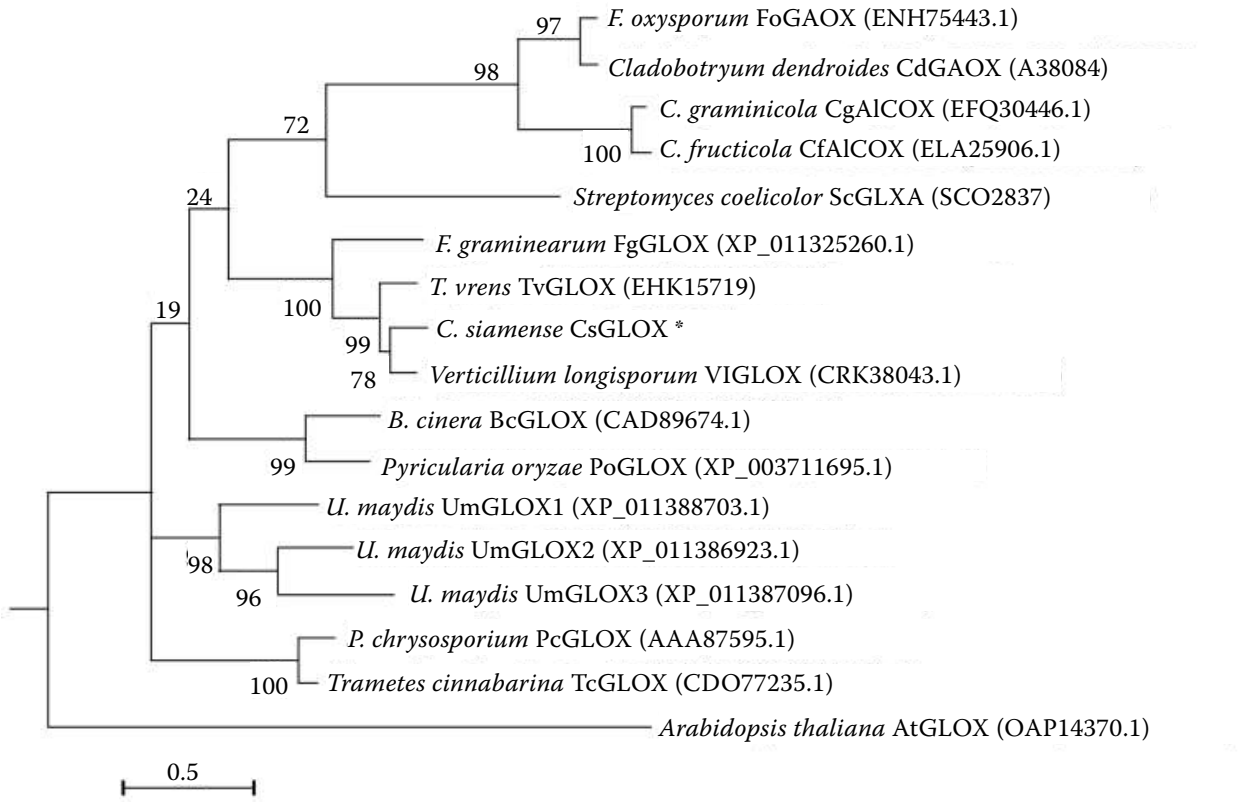

Figure 2. Phylogenetic tree of GLOXs and galactose oxidases
The phylogenetic tree was constructed based on the relevant amino acid sequences from different species by using the maximum likelihood method. CsGLOX is marked with a blue asterisk

(Crutcher et al. 2019). Moreover, Crutcher et al. (2019) suggested that *TvGLOX* also plays a critical role in conidial sporulation. Therefore, phenotypes such as those resulting from hyphal development, conidial sporulation, conidial, and appressorial morphology were evaluated to determine the effect of *CsGLOX* deficiency on the development of *C. siamense*.

For hyphal development analysis, conidial suspensions of WT and *CsGLOX*-deficient mutant strains D21 and D28 were inoculated onto PDA, CM, and MM solid medium, and the diameter of hyphal growth was observed after culture at 28 ℃ for 3 days. The colony diameter between WT and *CsGLOX*-deficient mutant strains on PDA solid media did not display a significant difference, and the same results were present on CM or MM solid medium (Figure 3B). These results indicated that the *CsGLOX* gene is not essential for the filamentous growth of *C. siamense*.

In order to examine the effects of *CsGLOX* gene on conidial sporulation, the conidia of WT, D21, and D28 were individually collected from 6-day-old PDA agar solid media. Then, the number and morphology of conidia were analyzed. The *CsGLOX*-deficient mutant strains D21 and D28 had significantly greater sporulation than WT (Figure 3C); however, *CsGLOX* deficiency did not affect the conidial morphology (Figure 3D). Furthermore, the appressorial morphology was observed by inoculating conidial suspensions onto paraffin-coated glass slides for 8 hours. The appressorial formation and morphology were not affected by *CsGLOX* (Figure 3D). These results indicated that *CsGLOX* is a major contributor to conidial sporulation.

***CsGLOX* gene contributes to the virulence of *C. siamense*.** As indicated by previous studies, plant pathogens' glyoxal oxidase can function in pathogenic virulence (Kan et al. 2004; Leuthner et al. 2005; Song et al. 2016). To assess the contribution of *CsGLOX* gene to the virulence of *C. siamense*, conidial suspension droplets of WT, D21, and D28 were inoculated onto detached young rubber tree leaves wounded with needles. The results showed that *CsGLOX* deficiency significant-

https://doi.org/10.17221/52/2023-PPS

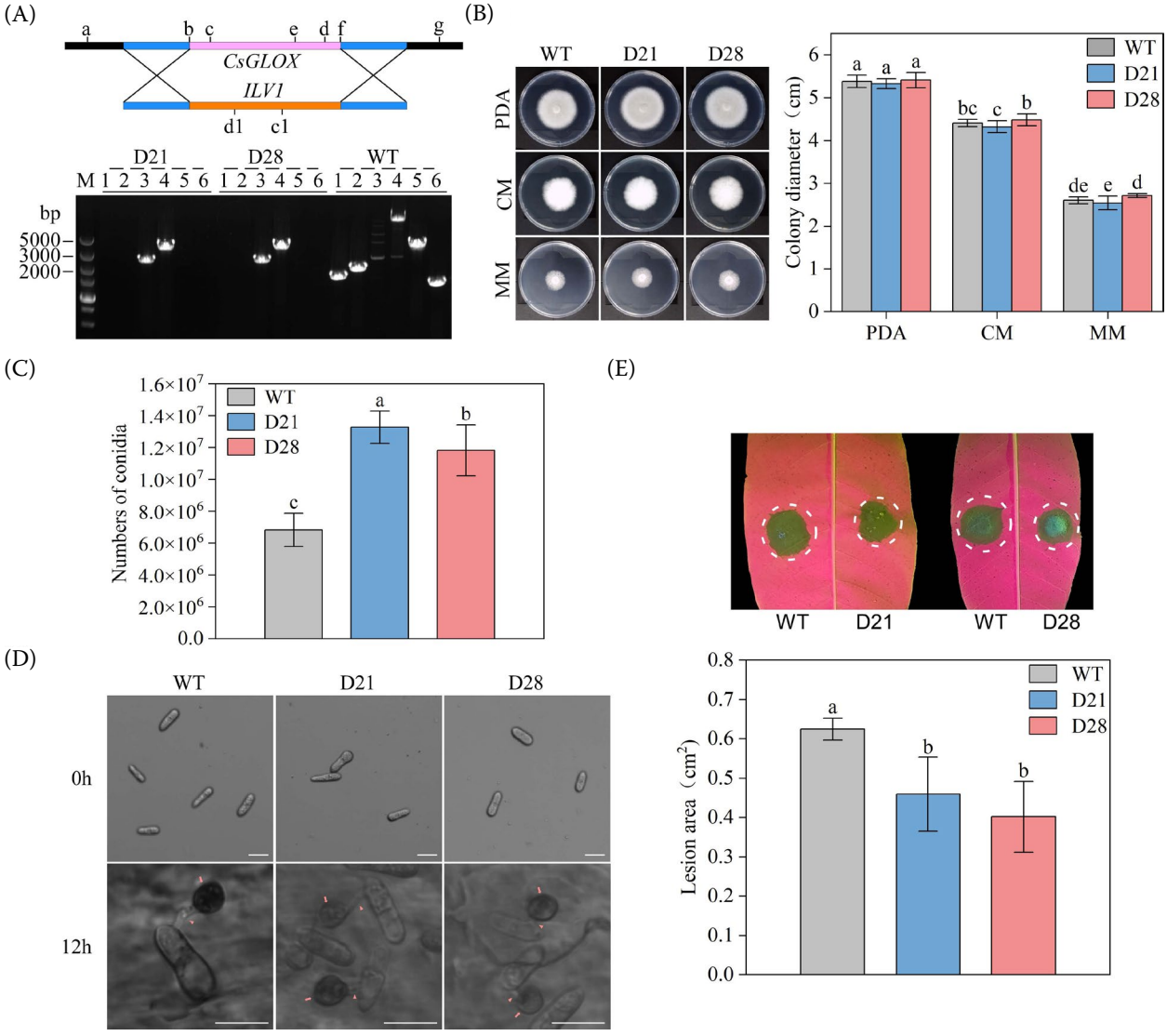

Figure 3. Effect of *C. siamense CsGLOX* gene on hyphal growth, conidia formation and pathogenicity

A – verification of *CsGLOX* gene knockout mutant of *Colletotrichum siamense*. M – DL 5000 DNA marker; 1 – using a/c as the verification primer; 2 – using d/g as the verification primer; 3 – using a/c1 as the verification primer; 4 – using d1/g as the verification primer; 5 – using b/f as the verification primer; 6 – using e/f as the verification primer

B – observation on mycelial growth of *C. siamense CsGLOX* gene knockout mutant on different media; PDA – potato dextrose agar medium; CM – complete medium; MM – minimal medium; each treatment contained three technical replicates, and each experiment was repeated three times (n = 9); WT – wild-type strain; D21/D28 – *CsGLOX* gene knockout mutant strains

C – effect of *C. siamense CsGLOX* gene on conidia yield; eight biological repeats were performed (n = 8)

D – effect of *C. siamense CsGLOX* gene on conidial and appressorial morphology; red triangle – germ tube; red arrow – appressorium; h – hours; bar = 10 μm

E – determination of the pathogenicity of *CsGLOX* gene knockout mutant strains of *C. siamense*; ten replicates were performed for each strain (n = 10); all the data are mean ± SE; one-way ANOVA with Tukey's multiple mean comparisons test method was used for data analysis; different lowercase letters indicate $P \leq 0.05$

ly reduced the ability of *C. siamense* to infect the rubber tree leaves, as shown by the smaller lesion areas produced by the two *CsGLOX*-deficient mutants D21 (mean 0.43 cm^2) and D28 (mean

https://doi.org/10.17221/52/2023-PPS

0.41 cm^2) compared with the wild-type strains (mean 0.63 cm^2) (Figure 3E).

***CsGLOX* gene plays a critical role in hyper-osmotic tolerance.** WSC domain have been implicated in the maintenance of cell wall integrity and stress response (Lodder et al. 1999; Futagami et al. 2011; Tong et al. 2016; Tong et al. 2019). Therefore, experiments were conducted on the ability of *CsGLOX* gene to respond to stress, as CsGLOX had five WSC domains at the N-terminus (Figure 1B). The sensitivity of WT, D21, and D28 was tested on PDA media supplemented with 2 mmol/L H_2O_2, 0.6 mol/L NaCl, 1 mol/L sorbitol and 6 µg/mL congo red. Under hyper-osmotic stress conditions, the D21 and D28 strains exhibited higher sensitivity than the WT strain, as the growth inhibition of Δ*CsGLOX* strains exposed to NaCl and sorbitol was remarkably increased compared to the WT strain under the corresponding conditions (Figure 4). On the contrary, the Δ*CsGLOX* strains showed slight resistance to congo red stress conditions compared to the WT strain (Figure 4), indicating that the *CsGLOX* gene may slightly negatively regulate the maintenance of cell wall integrity. A puzzling observation was that under H_2O_2 stress, D28 strains showed a significant increase in oxidative stress sensitivity, but D21 did not (Figure 4). Considering that D21 and D28 exhibited good phenotypic consistency under other stress conditions, we believe that the *CsGLOX* gene may not be involved in oxidative stress response.

DISCUSSION

Previous studies have clarified that GLOXs from saprotroph fungi (wood rot fungi) are mainly involved in lignin degradation (Bak et al. 2009; MacDonald et al. 2011; Kersten and Cullen 2014; Kadowaki et al. 2018). However, only a few *GLOXs* genes of phytopathogenic fungi, such as biotrophic fungi (*U. maydis*) and necrotrophic fungi (*F. graminearum* and *B. cinerea*), have been identified and functionally validated (Kan et al. 2004; Leuthner et al. 2005; Song et al. 2016). Therefore, the identification and functional characterization of *GLOXs* genes in hemi-biotrophic fungi are important for further understanding *GLOXs* genes' function in phytopathogenic fungi.

AA5_1 glyoxal oxidase gene is present in the genomes of different phytopathogenic fungi, and three types of GLOX proteins have been identified by protein structural domain analysis: *(i)* those only with AA5_ 1

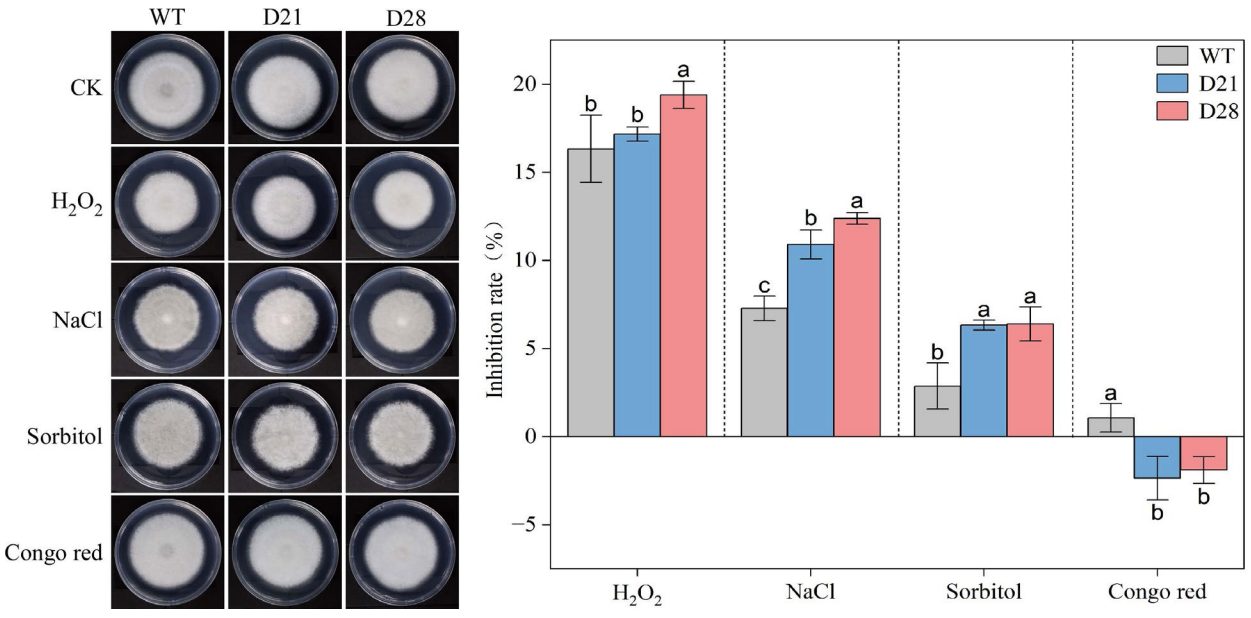

Figure 4. Effects of different stress factors on the mycelial growth of wild-type and *CsGLOX*-deficient strains of *Colletotrichum siamense*

Each treatment contained three technical replicates, and each experiment was repeated three times (n = 9); WT – wild-type strain; D21/D28 – *CsGLOX* gene knockout mutant strains; data are mean ± SE; one-way ANOVA with Tukey's multiple mean comparisons test method was used for data analysis; different lowercase letters indicate $P \leq 0.05$

https://doi.org/10.17221/52/2023-PPS

domain; *(ii)* those with N-terminal chitin-binding domains (ChtBD) and C-terminal AA5_ 1 domain; and *(iii)* those with N-terminal WSC domain and C-terminal AA5_ 1 domain (Kadowaki et al. 2018). In this study, protein structural domain analysis showed that the GLOX protein of the hemi-biotrophic fungus *C. siamense* has five N-terminal WSC structural domains in addition to the C-terminal catalytic structural domain AA5_1 (Figure 3B). The WSC domain, with its carbohydrate-binding properties, plays an important role in protein attachment to plant and/or fungal cell walls (Crutcher et al. 2019; Oide et al. 2019) and is associated with the maintenance of fungal cell wall integrity, as well as being involved in stress responses (Lodder et al. 1999; Futagami et al. 2011; Tong et al. 2016; Tong et al. 2019). Among the GLOXs with WSC domains, only TvGLOX from *T. virens* elucidated an important role for its WSC domain in binding carbohydrates (Crutcher et al. 2019), whereas whether it plays a role in the maintenance of fungal cell wall integrity and stress response is unclear. Here, we found that *CsGLOX* gene plays a critical role in hyper-osmotic tolerance (Figure 4), which is probably due to the contribution of its WSC domain, as there are no reports on the functional aspects of the AA5_1 domain involved in the regulation of fungal hyperosmotic tolerance. Additionally, we found that the *CsGLOX* gene may not contribute to maintaining fungal cell wall integrity. This is because under congo red (used to detect fungal cell wall integrity) stress conditions (Lima et al. 2021), the Δ*CsGLOX* strains showed slight resistance to congo red stress conditions compared to the WT strain (Figure 4). Thus, the *CsGLOX* gene may contribute to the hyperosmotic tolerance of the fungus rather than maintaining the integrity of the fungal cell wall.

CsGLOX gene deletion mutants were also used to characterize the effect of the *CsGLOX* gene on fungal mycelial growth and conidial formation (Figure 3B and 3C). Interestingly, the effects of the *CsGLOX* gene on mycelial growth and conidial production were different from those previously reported for other glyoxal oxidases (Kan et al. 2004; Leuthner et al. 2005; Crutcher et al. 2019). Unlike the reported glyoxal oxidases from biotrophic fungi (*U. maydis*) (Leuthner et al. 2005), necrotrophic fungi (*B. cinerea*) (Kan et al. 2004) and biological control fungi (*T. virens*) (Crutcher et al. 2019), deletion of the *CsGLOX* gene in the hemi-biotrophic fungus *C. siamense* not only did not affect mycelial growth (Figure 3B) and the appressorial formation (Figure 3D), but also led to a significant increase in conidial formation (Figure 3C). The reasons for this phenotype are unclear, but this phenotype may indicate that *CsGLOX* of the hemi-biotrophic fungus *C. siamense* may be more involved in pathogen-host interactions than in influencing the growth and morphology of its hyphae, as there is increasing evidence that GLOXs from plant pathogenic fungi are extracellular proteins involved in pathogen-plant interactions (Meinhardt et al. 2014; González-Fernández et al. 2015; Vela-Corcía et al. 2016; Lopez et al. 2018; Haile et al. 2020; Ribeaucourt et al. 2021; Chandrasekar et al. 2022).

It has been shown in previous studies that the *GLOXs*-deficient in the biotrophic phytopathogenic fungus *U. maydis* and the necrotrophic phytopathogenic fungus *B. cinerea* results in complete non-pathogenicity (Kan et al. 2004; Leuthner et al. 2005). Interestingly, the *CsGLOX*-deficient strains of *C. siamense* significantly reduced their virulence in plants but did not completely lose pathogenicity (Figure 3E). Other WSC domain-containing glyoxal oxidases (from *F. verticillioides* and *F. oxysporum*) have been previously studied, and they had similar effects on pathogenicity (Crutcher et al. 2019). One of the known functions of GLOX is participating in the degradation of lignin by providing H_2O_2 for the oxidation reaction catalyzed by extracellular peroxidase (Kersten et al. 1995; Bak et al. 2009; Takano et al. 2010; MacDonald et al. 2011; Kersten & Cullen 2014; Matityahu et al. 2015; Kadowaki et al. 2018; Wohlschlager et al. 2021). Therefore, to explain the pathogenicity defect of *CsGLOX* gene mutants, we suggest that H_2O_2 production by CsGLOX activity may be one of the necessary requirements for infection. Since the *C. siamense* genome has extracellular peroxidases involved in lignin degradation, such as ligninase and manganese peroxidase (data are not shown). Additionally, previous studies have shown that H_2O_2 plays an important role in influencing the lifestyle of *C. gloeosporioides* during its interaction with the host (Eloy et al. 2015). When increasing plant H_2O_2 accumulation and lipid peroxidation, the fungus employs a subcuticular, intramural necrotrophic strategy and develops secondary hyphae associated with rapid dispersal and rapid killing of host cells. However, when decreasing plant H_2O_2 concentration and lipid peroxidation, the fungus developed intracellular hemi-biotrophic infection with vesicles and primary and secondary hyphal formation (Eloy et al. 2015). Thus, we can also envision a scenario in which *CsGLOX* deficiency reduces the accumulation of H_2O_2 in plant cells, leading to a reduction in the production of secondary mycelium associated with rapid pathogen spread and rapid killing of host cells, which in turn reduces pathogenicity.

https://doi.org/10.17221/52/2023-PPS

CONCLUSION

In conclusion, this study identified a GLOX-encoding gene *CsGLOX* from hemi-biotrophic fungi *C. siamense,* and the results of the *CsGLOX* knockout assay suggest that the *CsGLOX* gene plays an important role in conidial formation, hyperosmotic stress tolerance and pathogenicity. Subsequent studies will focus on the effect of the WSC structural domain on CsGLOX enzyme activity, as well as its function in high osmotic stress tolerance and influence on the pathogenicity of phytopathogenic fungi. Further functional studies of *GLOXs* genes in hemi-biotrophic fungi are essential for refining the function of *GLOXs* in phytopathogenic fungi.

REFERENCES

Bak J.S., Ko J.K., Choi I.G., Park Y.C., Seo J.H., Kim K.H. (2009): Fungal pretreatment of lignocellulose by *Phanerochaete chrysosporium* to produce ethanol from rice straw. Biotechnology and Bioengineering, 104: 471–482.

Cao X.R., Xu X.M., Che H.Y., West J.S., Luo D.Q. (2017): Distribution and fungicide sensitivity of *Colletotrichum* species complexes from rubber tree in Hainan, China. Plant Disease, 101: 1774–1780.

Chandrasekar B., Wanke A., Wawra S., Saake P.., Mahdi L., Charura N., Neidert M., Poschmann G., et al. (2022): Fungi hijack a ubiquitous plant apoplastic endoglucanase to release a ROS scavenging β-glucan decasaccharide to subvert immune responses. Plant Cell, 34: 2765–2784.

Crutcher F.K., Moran-Diez M.E., Krieger I.V., Kenerley C.M. (2019): Effects on hyphal morphology and development by the putative copper radical oxidase glx1 in *Trichoderma virens* suggest a novel role as a cell wall associated enzyme. Fungal Genetics and Biology, 131: 103245. doi: 10.1016/j.fgb.2019.103245

Daou M., Faulds C.B. (2017): Glyoxal oxidases: their nature and properties. World journal of Microbiology and Biotechnology, 33: 87.

Eloy Y.R.G., Vasconcelos I.M., Barreto A.L.H., Freire-Filho F.R., Oliveira J.T.A. (2015): H_2O_2 plays an important role in the lifestyle of *Colletotrichum gloeosporioides* during interaction with cowpea [*Vigna unguiculata* (L.) Walp]. Fungal biology, 119: 747–757.

Fong J.K., Brumer H. (2023): Copper radical oxidases: galactose oxidase, glyoxal oxidase, and beyond. Essays in Biochemistry, 67: 597–613.

Futagami T., Nakao S., Kido Y., Oka T., Kajiwara Y., Takashita H., Omori T., Furukawa K., et al. (2011): Putative stress sensors WscA and WscB are involved in hypo-osmotic and acidic pH stress tolerance in *Aspergillus nidulans*. Eukaryotic cell, 10: 1504–1515.

González-Fernández R., Valero-Galván J., Gómez-Gálvez F.J., Jorrín-Novo Jesús V.J. (2015): Unraveling the *in vitro* secretome of the phytopathogen *Botrytis cinerea* to understand the interaction with its hosts. Frontiers in Plant Science, 6: 839.

Haile Z.M., Malacarne G., Pilati S., Sonego P., Moretto M., Masuero D., Vrhovsek U., Engelen K., et al. (2020): Dual transcriptome and metabolic analysis of *Vitis vinifera* cv. pinot noir berry and *Botrytis cinerea* during quiescence and egressed infection. Frontiers in Plant Science, 10: 1704.

Kadowaki M.A.S., Godoy M.O.D., Kumagai P.S., Costa-Filho A.J.D., Mort A., Prade R.A., Polikarpov I. (2018): Characterization of a new glyoxal oxidase from the thermophilic fungus *Myceliophthora thermophila* M77: Hydrogen peroxide production retained in 5-hydroxymethylfurfural oxidation. Catalysts, 8: 476.

Kan J.A.L.V., Stefanato F., Raats E.M. (2004): A mysterious, pleiotropic growth defect caused by deletion of a glyoxal oxidase gene in *Botrytis cinerea*. In: Book of Abstracts 7th European Conference on Fungal Genetics, April 17–20, 2004, Copenhagen, Denmark: 168–168.

Kersten P., Cullen D. (2014): Copper radical oxidases and related extracellular oxidoreductases of wood-decay Agaricomycetes. Fungal Genetics and Biology, 72: 124–130.

Kersten P.J., Kirk T.K. (1987): Involvement of a new enzyme, glyoxal oxidase, in extracellular H_2O_2 production by *Phanerochaete chrysosporium*. Journal of Bacteriology, 169: 2195–2201.

Kersten P.J., Witek C., Wymelenberg A.V., Cullen D. (1995): *Phanerochaete chrysosporium* glyoxal oxidase is encoded by two allelic variants: structure, genomic organization, and heterologous expression of glx1 and glx2. Journal of Bacteriology, 17: 6106–6110.

Koschorreck K., Alpdagtas S., Urlacher V.B. (2022): Copper-radical oxidases: A diverse group of biocatalysts with distinct properties and a broad range of biotechnological applications. Engineering Microbiology, 2: 100037. doi: 10.1016/j.engmic.2022.100037

Leuthner B., Aichinger C., Oehmen E., Koopmann E., Müller O., Müller P., Kahmann R., Bölker M., et al. (2005): A H_2O_2-producing glyoxal oxidase is required for filamentous growth and pathogenicity in *Ustilago maydis*. Molecular Genetics and Genomics, 272: 639–650.

Lima D.M.C.G., Costa T.P.C., Emri T., Pócsi I., Pupin B., Rangel D.E.N. (2021): Fungal tolerance to Congo red, a cell wall integrity stress, as a promising indicator of ecological niche. Fungal Biology, 125: 646–657.

Lin C.H., Huang G.X., Zheng F.C., Miao W.G. (2018): Functional characterization of CgPBS2, a MAP kinase in *Colletotrichum gloeosporioides*, using osmotic stress sensitivity

https://doi.org/10.17221/52/2023-PPS

as a selection marker. Eeuropean Journal Plant Pathology, 152: 801–813.

Liu X.B., Li B.X., Cai J.M., Zheng Z.L., Feng Y.L., Huang G.X. (2018): *Colletotrichum* species causing anthracnose of rubber trees in China. Scientific Reports, 8: 10435.

Liu X.B, Li B.X., Yang Y., Cai J.M., Shi T., Zheng X.L., Huang G.X. (2020): Pathogenic adaptations revealed by comparative genome analyses of two *Colletotrichum* spp., the causal agent of anthracnose in rubber tree. Frontiers in Microbiology, 11: 1484.

Lodder A.L., Lee T.K., Ballester R. (1999): Characterization of the Wsc1 protein, a putative receptor in the stress response of *Saccharomyces cerevisiae*. Genetics, 152: 1487–1499.

Lopez D., Ribeiro S., Label P., Fumanal B., Venisse J.S., Kohler A., Oliveira R.R.D., Labutti K., et al. (2018): Genome-wide analysis of *Corynespora cassiicola* leaf fall disease putative effectors. Frontiers in Microbiology, 9: 276.

MacDonald J., Doering M., Canam T., Gong Y.C., Guttman D.S., Campbell M.M., Master E.R. (2011): Transcriptomic responses of the softwood-degrading whiterot fungus *Phanerochaete carnosa* during growth on coniferous and deciduous wood. Applied and Environmental Microbiology, 77: 3211–3218.

Matityahu A., Sitruk A., Hadar Y., Belinky P.A. (2015): Factors affecting the induction of lignin peroxidase in manganese-deficient cultures of the white rot fungus *Phanerochaete chrysosporium*. Advances in Microbiology, 5: 83–92.

Meinhardt L.W., Costa G.G.L., Thomazella D.P., Teixeira P.J.P., Carazzolle M.F., Schuster S.C., Carlson J.E., Guiltinan M.J., et al. (2014): Genome and secretome analysis of the hemibiotrophic fungal pathogen, *Moniliophthora roreri*, which causes frosty pod rot disease of cacao: mechanisms of the biotrophic and necrotrophic phases. BMC Genomics, 15: 164.

Oide S., Tanakaa Y., Watanabea A., Inui M. (2019): Carbohydrate-binding property of a cell wall integrity and stress response component (WSC) domain of an alcohol oxidase from the rice blast pathogen *Pyricularia oryzae*. Enzyme and Microbial Technology, 125: 13–20.

Ribeaucourt D., Saker S., Navarro D., Bissaro B., Drula E., Correia L.O., Haon M., Grisel S., et al. (2021): Identification of copper-containing oxidoreductases in the secretomes of three *Colletotrichum* species with a focus on copper radical oxidases for the biocatalytic production of fatty aldehydes. Applied and Environmental Microbiology, 87: e01526–21. doi: 10.1128/AEM.01526-21

Robert X., Gouet P. (2014): Deciphering key features in protein structures with the new ENDscript server. Nucleic Acids Rresearch, 42: 320–324.

Song M., Fang S.Q., Li Z.G., Wang N., Li X., Liu W.B., Zhang Y., Lin C.H., et al. (2022): CsAtf1, a bZIP transcription factor, is involved in fludioxonil sensitivity and virulence in the rubber tree anthracnose fungus *Colletotrichum siamense*. Fungal Genetics and Biology, 158: 103649.

Song X.S., Xing S., Li H.P., Zhang J.B., Qu B., Jiang J.H., Fan C., Yang P., et al. (2016): An antibody that confers plant disease resistance targets a membrane-bound glyoxal oxidase in *Fusarium*. New Phytologist, 210: 997–1010.

Takano M., Nakamura M., Yamaguchi M. (2010): Glyoxal oxidase supplies hydrogen peroxide at hyphal tips and on hyphal wall to manganese peroxidase of white-rot fungus *Phanerochaete crassa* WD1694. Journal of Wood Science, 56: 307–313.

Tong S.M., Chen Y., Zhu J., Ying S.H., Feng M.G. (2016): Subcellular localization of five singular WSC domain-containing proteins and their roles in *Beauveria bassiana* responses to stress cues and metal ions. Environmental Microbiology Reports, 8: 295–304.

Tong S.M., Wang D.Y., Gao B.J., Ying S.H., Feng M.G. (2019): The DUF1996 and WSC domain-containing protein Wsc1I acts as a novel sensor of multiple stress cues in *Beauveria bassiana*. Cellular Microbiology, 21: e13100. doi: 10.1111/cmi.13100

Vela-Corcía D., Bautista R., Vicente A.D., Spanu P.D., Pérez-García A. (2016): De novo analysis of the epiphytic transcriptome of the cucurbit powdery mildew fungus *Podosphaera xanthii* and identification of candidate secreted effector proteins. Plos One, 11: e0163379. doi: 10.1371/journal.pone.0163379

Wohlschlager L., Kracher D., Scheiblbrandner S., Csarman F., Ludwig R. (2021): Spectroelectrochemical investigation of the glyoxal oxidase activation mechanism. Bioelectrochemistry, 141: 107845. doi: 10.1016/j.bioelechem.2021.107845

Yin D.T., Urresti S., Lafond M., Johnston E.M., Derikvand F., Ciano L., Berrin J.G., Henrissat B., et al. (2015): Structure-function characterization reveals new catalytic diversity in the galactose oxidase and glyoxal oxidase family. Nature Communications, 6: 10197.

Received: May 9, 2023
Accepted: October 4, 2023
Published online: November 10, 2023