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Glutathione peroxidase LtGPX3 contributes to oxidative stress tolerance, virulence, and plant defense suppression in the peach gummosis fungus *Lasiodiplodia theobromae*

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Abstract

The notorious woody plant-degrading pathogen *Lasiodiplodia theobromae* is a major causal agent of peach gummosis, one of the prevalent and devastating trunk diseases to peach production; however, its pathogenesis is largely unknown. Our previous study showed that *L. theobromae LtGPX3*, which encodes a glutathione peroxidase resembling yeast GPX3/HYR1-like, was constantly and dramatically upregulated at the infectious stages. Here, we functionally characterized *LtGPX3* using the CRISPR-Cas9-aided split marker approach. The *ΔLtgpx3* deletion mutants displayed increased sensitivity to the osmotic stress agent KCl and less sensitivity to the cell wall-damaging agent calcofluor white. Exogenous oxidants highly induced the expression of *LtGPX3*, and the *ΔLtgpx3* mutants displayed increased sensitivity to ROS-generating oxidants. Pathogenicity assays revealed that *ΔLtgpx3* mutants showed compromised virulence in peach shoots, which was partially restored when peach shoots were pretreated with an NADPH oxidase inhibitor before inoculation. Moreover, ROS levels were strongly boosted, and transcripts of plant defense-related genes were highly induced in the *ΔLtgpx3* mutants-infected peach shoots compared with the wild-type-inoculated. Overall, our results showed the essential roles of *LtGPX3* in the oxidative stress response and tolerance and pathological functions in *L. theobromae*. These findings deepen our understanding of the survival strategies of the woody plant-degrading pathogen *L. theobromae* and provide new insights into developing new strategies for peach gummosis disease control.

Keywords Fungal virulence, Homologous recombination, Reactive oxygen species, Plant defense response, *Prunus persica*, Woody tree disease

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Background

The notorious woody plant-degrading pathogen *Lasiodiplodia theobromae* is a latent or opportunistic fungus and has been reported to attack ~ 500 fruit and woody trees, leading to gum exudation, canker, dieback, and fruit rot in many economically valuable fruit and forest trees (Peng et al. 2022). *L. theobromae* is a known pathogen causing peach gummosis, one of the major constraints to peach production, especially in the United States, China, and Japan, leading to enormous reductions in tree vigor and commercial production (Wang et al. 2011). Several fungicides have been used to control this disease; however, the incidence of fungicide resistance is increasing (Beckman et al. 2011; Zhang et al. 2022). Hence, a holistic exploration of the peach-*L. theobromae* interaction is of great importance in managing peach gummosis disease.

During infection, plants often produce mass reactive oxygen species (ROS), termed oxidative burst, which confer resistance against pathogens (Dos and Franco 2023; Feng et al. 2023). ROS, including superoxide (O_2^-) and hydrogen peroxide (H_2O_2), are typically generated by plasma membrane-localized NADPH oxidases (respiratory burst oxidase homologs, RBOHs). High accumulation of ROS causes damage to biomolecules, such as DNA, lipids, and proteins, and finally brings about programmed cell death (Khan et al. 2023; Yu et al. 2023). However, ROS also act as signaling molecules and can trigger an array of plant defense responses, such as boosting defense-related gene transcription and reinforcing cell wall structures, to cope with pathogen invasion (Singh et al. 2021). Our previous studies revealed that ROS burst occurs during *L. theobromae* infection and the transcription of ROS-related defense genes is highly upregulated, suggesting that ROS-mediated plant defense responses contribute to constrain *L. theobromae* proliferation and spreading around the infection sites (Zhang et al. 2021; Zhang et al. 2020). Consequently, ROS signaling plays a pivotal role in the pathogenesis of peach gummosis disease.

To overcome oxidative stress and achieve successful colonization, phytopathogens have evolved a plethora of intricate strategies for ROS detoxification (Segal and Wilson 2018; Vallières et al. 2023). The yeast-activating protein 1 (YAP1) of budding yeast *Saccharomyces cerevisiae* and its orthologs in fungal pathogens act as core transcriptional regulators of oxidative stress-responsive and antioxidant genes, such as glutathione genes, which are important to neutralize extra ROS (Mendoza-Martínez et al. 2020; Singh et al. 2021; Yaakoub et al. 2022). Our previous studies have demonstrated that the infection by *L. theobromae* leads to a ROS burst; meanwhile, the expressions of *LtAP1* (activating protein 1) and glutathione genes are consistently and dramatically induced in *L. theobromae* (Zhang et al. 2020). Moreover, the transcription levels of glutathione genes are severely reduced when *LtAP1* is mutated during *L. theobromae* infection into peach shoots (Zhang et al. 2021). These findings demonstrate that *LtAP1* may participate in ROS neutralization by activating the glutathione system. Hence, we assume that the glutathione system is crucial in regulating ROS homeostasis and oxidative stress tolerance in *L. theobromae.*

In eukaryotic cells, glutathione peroxidase (GPX), which catalyses the reduction of H_2O_2 to water, is one of the primary defense responses against H_2O_2-derived cellular damage (Fichman et al. 2023; Waszczak et al. 2018). *S. cerevisiae GPX3* (also termed *HYR1*) is highly induced in the presence of oxidants, and the *GPX3/HYR1* mutant is hypersensitive to oxidants (Avery et al. 2004). In *Magnaporthe oryzae*, the GPX3/HYR1 ortholog is essential in oxidative stress tolerance and fungal virulence (Huang et al. 2011a). Similarly, the *AaGPX3* deletion mutants of *Alternaria alternata* show increased sensitivity to ROS and reduced virulence (Yang et al. 2016). A recent study shows that deletion of glutathione peroxidase gene *VpGP* in *Valsa mali* results in enhanced sensitivity to H_2O_2 and impaired virulence in apple shoots (Feng et al. 2021). Therefore, GPX plays a crucial role in pathogen infection, but its function in *L. theobromae* pathogenesis remains unknown.

In this study, we functionally characterized *L. theobromae* glutathione peroxidase gene *LtGPX3* through CRISPR-Cas9-aided gene deletion and investigated the effects of *LtGPX3* deletion on sensitivity to oxidative and other chemical stresses, fungal virulence, and plant defense response. This study sheds light on the roles of *LtGPX3* in oxidative stress, ROS neutralization, virulence, and plant defense response during *L. theobromae* infection of peach shoots. Our findings provide important insights into the essential role of ROS detoxification in the virulence of the notorious woody plant-degrading pathogen *L. theobromae*, which could enrich our understanding of fungal phytopathogen survival strategies and provide new implications for the control of fungal diseases.

Results

Identification of *L. theobromae LtGPX3*

L. theobromae glutathione peroxidase gene *LtGPX3* (accession number: MN933616.1) contains a 504 bp coding sequence with a 461 bp intron and is predicted to encode a polypeptide with 167 amino acids. The LtGPX3 amino acid sequence showed 87% identity and 94% similarity to the GPX3 homologue of *Diplodia seriata* (accession number: OMP87767.1) in the National Center for

Biotechnology Information (NCBI) database. LtGPX3 exhibits 78% identity and 87% similarity to AaGPX3 of *A. alternata* (accession number: ACY73852.1) (Additional file 1: Figure S1). A PROSITE search and multiple sequence alignment show that LtGPX3 has the highly conserved glutathione peroxidase active site (GKvVLVvNTaSkCGfT) and glutathione peroxidase signature (LGFPCNQF) (Fig. 1a, b). Additionally, three conserved cysteines (Cys39, Cys70, and Cys88) are found in LtGPX3 (Fig. 1b). Moreover, the predicted 3D structural analysis shows that LtGPX3 shares a robust similarity with chain A of the crystal structure of glutathione-dependent phospholipid peroxidase HYR1 of yeast *S. cerevisiae* (Fig. 1c).

Genetic modification of *LtGPX3*

To functionally characterize *LtGPX3*, we generated its deletion and complementation strains through homologous recombination and CRISPR/Cas9 approaches (Fig. 2a, b). Primers 16F/16R and 17F/17R amplified two PCR products of 2501 and 2697 bp, respectively, from genomic DNA of the deletion mutants *ΔLtgpx3*, whereas no product was detected from the wild-type (WT; Fig. 2c). Furthermore, the *ΔLtgpx3* transformants was also verified by PCR using primers 15F/15R and by growing on hygromycin plates (Fig. 2c and Additional file 1: Figure S2). Also, the transcripts of *LtGPX3* were not detected in two *ΔLtgpx3* mutants (Additional file 1: Figure S3). These results indicate successful deletion of the *LtGPX3* gene in *L. theobromae*, and the two independent *ΔLtgpx3* mutants were used for further analysis (Fig. 2c).

The complementation transformant *ΔLtgpx3/*GPX3 was obtained by transforming the *ΔLtgpx3* mutant with the native promoter-driven *LtGPX3::NEO* construct. An 824 bp PCR product was amplified from the genomic DNA of the complementation transformants using primers 15F/15R, while no product was amplified from the deletion transformants (Fig. 2c). We thus obtained five complementation transformants and randomly chose one for further studies.

LtGPX3 deficiency reshaped *L. theobromae* stress response

To evaluate the role of *LtGPX3* in *L. theobromae* response to exogenous stresses, *LtGPX3* deletion and complementation strains were treated with various stresses. The *ΔLtgpx3* mutants exhibited increased sensitivity to KCl compared with the WT (Fig. 3). In contrast, mycelial growth of the *ΔLtgpx3* mutants was significantly enhanced in calcofluor white (CFW)- and sorbitol-containing potato dextrose agar (PDA) plates compared to the WT (Fig. 3). Mycelial morphology and colony diameter of the *ΔLtgpx3/*GPX3 strain under stress treatments were rescued (and even increased in the sorbitol treatment) to WT levels (Fig. 3).

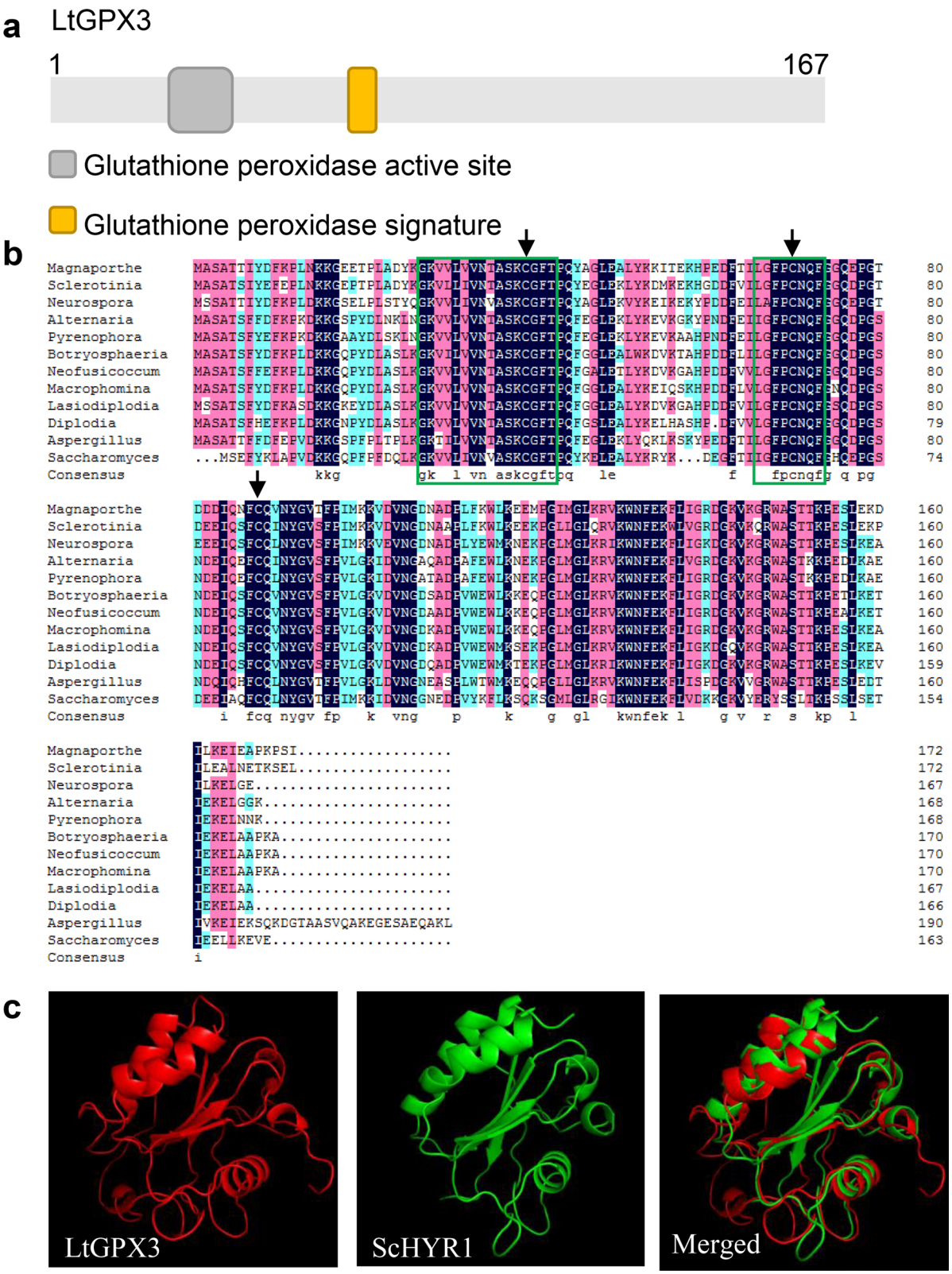
We also investigated the response of *LtGPX3* to oxidative stress. Mycelial plugs of the three genotypes were cultured on PDA plates containing various oxidative stress reagents, including 5 mM H_2O_2, 0.68 mM cumene H_2O_2, 0.5 mM *tert*-butyl-hydroperoxide (TBHP), and 5 mM sodium nitroferricyanide dihydrate (SNP). Compared to the WT strain, two *ΔLtgpx3* mutants increased sensitivity to all the tested oxidative reagents (Fig. 4a, b). Further, transcript levels of *LtGPX3* in the WT strain under H_2O_2 or TBHP treatments were analyzed by quantitative reverse-transcription PCR (qRT-PCR). When exposed to H_2O_2, transcript levels of *LtGPX3* dramatically increased at 15 min and peaked at 45 min, followed by a slight reduction at 60 min, compared with untreated mycelia (0 min) (Fig. 4c). Similarly, transcript levels of *LtGPX3* were also significantly upregulated at all time points and peaked at 45 min under TBHP treatment (Fig. 4d).

LtGPX3 deficiency reduced fungal virulence in peach shoots

To determine the role of *LtGPX3* in virulence, we performed disease assays on detached current-year peach shoots. The shoots showed large and oval lesions with visible gum release after inoculation with WT or the complementation strain *ΔLtgpx3/*GPX3. Conversely, the *ΔLtgpx3* mutants caused reduced necrotic lesions or negligible gum release compared with WT and *ΔLtgpx3/*GPX3 strains (Fig. 5a, b). Quantitative analysis showed that the average lesion size on the *ΔLtgpx3* mutants-inoculated peach shoots was 37% less than that on the WT strain-inoculated (Fig. 5b). Furthermore, the fungal biomass in shoots inoculated with *ΔLtgpx3* mutants was significantly lower than in shoots inoculated with WT- and *ΔLtgpx3/*GPX3 strains (Fig. 5c).

(See figure on next page.)

Fig. 1 LtGPX3 is a putative glutathione peroxidase protein highly conserved among filamentous fungi. **a** LtGPX3 has two glutathione peroxidase domains, active (gray box) and signature (orange box) sites, through the Prosite search. **b** Alignment of *Lasiodiplodia theobromae* LtGPX3 with orthologs from ten filamentous fungi and *Saccharomyces cerevisiae*. The green border shows the location of two domains as shown in **a**. Arrows indicate the conserved cysteines. **c** The 3D structural comparison between LtGPX3 (5 to 166 aa) and glutathione peroxidase-like peroxiredoxin HYR1 of *S. cerevisiae*



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Increased ROS accumulation in *LtGPX3* mutant-infected shoots

To test the function of *LtGPX3* in detoxifying ROS, we measured O_2^- and H_2O_2 contents in the *ΔLtgpx3* mutant- or WT-infected peach shoots at 5 days post-inoculation (dpi). The O_2^- and H_2O_2 levels were 1.9- and 1.1-fold higher in the *ΔLtgpx3* mutant-inoculated shoots than in the WT-infected shoots (Fig. 6a, b). Furthermore, to understand whether *LtGPX3* expression was associated with plant ROS production during infection, transcript levels of plant ROS biosynthetic genes *PpRBOHs* were analyzed in the shoots at 5 dpi. Results showed that the transcript levels of *PpRBOHD* and *PpRBOHF* in the *ΔLtgpx3* mutant-inoculated shoots were significantly upregulated by 1.6- and 1.3-fold, respectively, compared with the WT-inoculated shoots (Fig. 6c, d).

Suppression of ROS generation enhanced the virulence of *LtGPX3* mutant strains

To better understand the relationship between *LtGPX3*-mediated oxidative stress tolerance and fungal virulence, detached peach shoots were pre-sprayed with an NADPH oxidase inhibitor diphenylene iodonium (DPI) prior to the inoculation with *ΔLtgpx3* mutants. The DPI-treated shoots showed larger necrotic lesions and more visible gum release than the untreated control following infection (Fig. 7a, b). Quantitatively, the lesion diameter caused by *ΔLtgpx3* mutants without DPI treatment was found to be only 54% of the diameter observed with DPI treatment. However, DPI treatment alone did not cause lesions or gum release on wounded peach shoots (Fig. 7b).

Involvement of *LtGPX3* in repressing peach defense responses

Given the reduced lesions and increased ROS accumulation in *ΔLtgpx3*-inoculated shoots, we speculated that plant defense-related genes might be actively upregulated. Transcript levels of eight defense-related genes, including those involved in the salicylic acid (SA) biosynthesis pathway and pathogenesis-related (*PR*) genes, were examined in the infected peach shoots. Results showed that transcript levels of *PpICS1* (Isochorismate synthase 1), *PpPAL1* (Phenylalanine ammonia lyase 1), and *PpPR1a*, which are involved in SA pathways, were significantly upregulated in *ΔLtgpx3* mutant-inoculated shoots compared with WT-infected shoots (Fig. 8a–c). In addition, transcript levels of *PR* genes, including *PpPR4*, *PpTLP1* (Thaumatin-like protein 1, PR5 family), *PpPR8*, *PpPR10-1*, *PpPR10-4*, and *PpDFN1* (Defensin 1, PR12 family), were also significantly higher in the peach shoots infected by *ΔLtgpx3* mutants than in WT-infected tissues (Fig. 8d–i). Notably, levels of *PpPR4* and *PpDFN1* transcripts induced by *ΔLtgpx3* mutants were nearly three times higher than those induced by the WT strain (Fig. 8d, i). These observations suggest a positive role of *LtGPX3* in suppressing plant defense responses.

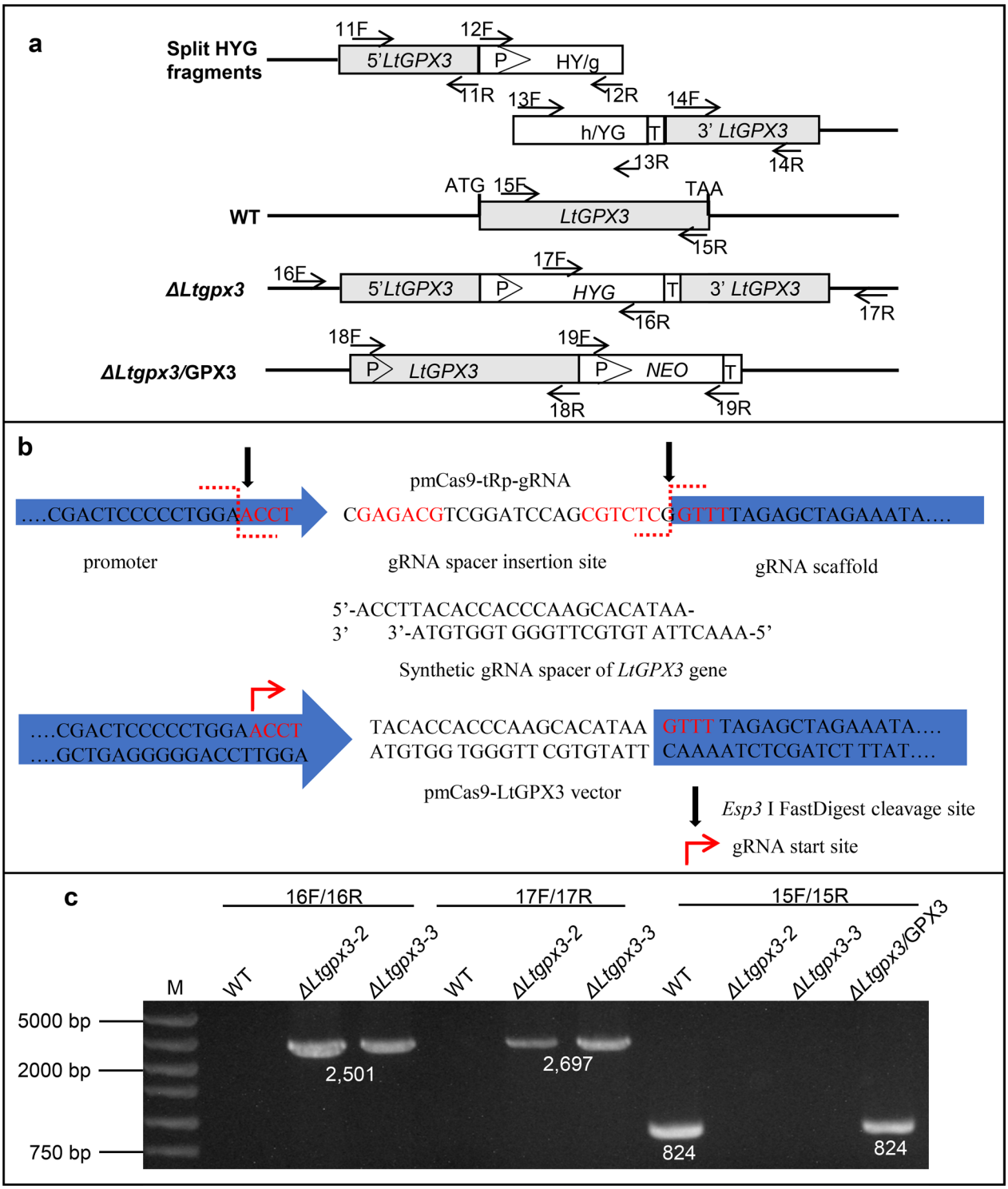
Discussion

L. theobromae LtGPX3, encoding a glutathione peroxidase, is involved in peach gummosis

The woody plant-degrading pathogen *L. theobromae* causes peach gummosis, a highly detrimental disease to peach production worldwide (Wang et al. 2011; Zhang et al. 2023). However, the pathogenicity of *L. theobromae* in peach gummosis at the molecular level is still largely unknown. A ROS burst occurs in *L. theobromae*-inoculated peach shoots, and the transcripts of several fungal oxidative stress response-related genes, such as *LtAP1* and *LtGPX3*, are consistently induced during *L. theobromae*-peach shoot interaction, indicating that these genes might play important roles in ROS neutralization (Zhang et al. 2020). Furthermore, *LtAP1*, a central regulator in activating glutaredoxin and thioredoxin systems, is involved in the oxidative stress response and virulence in *L. theobromae*-induced gummosis. In the *LtAP1* deletion mutant, the transcript level of *LtGPX3*, a core member of the glutaredoxin system, is markedly downregulated (Zhang et al. 2021), implying that *LtGPX3* might work in the oxidative stress response and even in fungal virulence.

(See figure on next page.)

Fig. 2 Targeted disruption of *LtGPX3* in *L. theobromae* using a CRISPR-Cas9-aided split marker approach. **a** Schematic illustration of the PCR strategy for generating split marker fragments overlapping within the hygromycin phosphotransferase gene (*HYG*) cassette under the control of the *Aspergillus nidulans trpC* gene promoter (P) and terminator (T) within *LtGPX3* (in shaded bars). *ΔLtgpx3/*GPX3 expressing LtGPX3 under the control of *LtGPX3* native promoter. The arrowheads indicate the primer locations and directions. **b** Schematic illustration of plasmid pmCas9-LtGPX3 construction. The nucleotides in red indicate the *Esp*3I enzyme digestion site. The gRNA spacers were synthesized by annealing the sense and antisense oligonucleotides with 5′-ACCT and AAAC-3′ overhangs and inserted into *Esp*3I-digested pmCas9 empty vector by T4 DNA ligase to generate plasmid pmCas9-LtGPX3. **c** PCR amplification of DNA fragments from genomic DNA of WT and two *ΔLtgpx3* mutants (*ΔLtgpx3-2* and *-3*) with indicated primers. Two pairs of primers (16F/16R and 17F/17R) were used to confirm *LtGPX3* deletion transformants. Primers 16F and 17R were used to examine in situ integration of *HYG* within the *LtGPX3* allele. Two *LtGPX3*-specific primers (15F/15R) amplified an expected 824 bp fragment from the genomic DNA of WT and *ΔLtgpx3/*GPX3 strains



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In this study, we cloned and functionally characterized the *L. theobromae LtGPX3* gene, which encodes a glutathione peroxidase resembling yeast GPX3/HYR1-like. Sequence analysis revealed that LtGPX3 contains two glutathione peroxidase domains: an active site and a signature domain—highly conserved domains commonly found in glutathione peroxidase orthologs of fungi (Fig. 1a, b). Two essential cysteine residues (Cys39 and

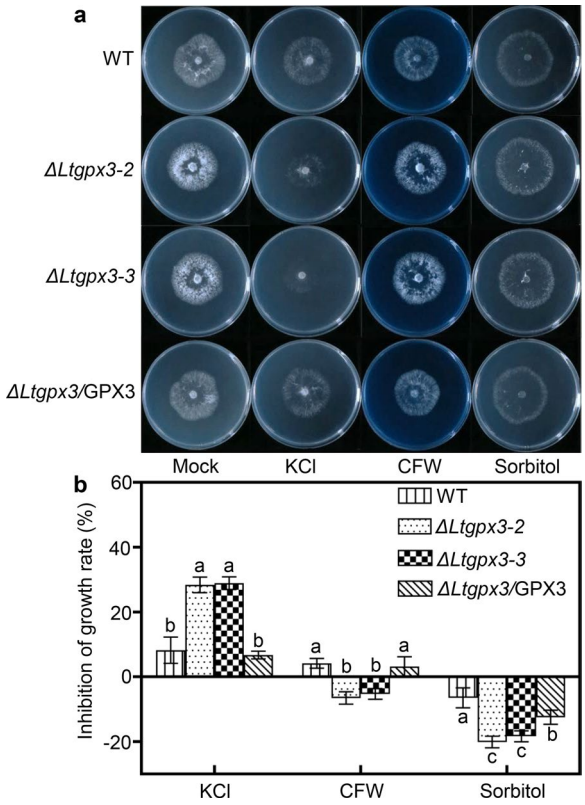

Fig. 3 Mycelial growth of the *Ltgpx3* mutants under different stress conditions. **a** Mycelial cultures of the WT, two *LtGPX3* deletion mutants (*ΔLtgpx3-2* and *-3*), and the complementation strain *ΔLtgpx3*/GPX3 grown on PDA media supplemented with 1 M KCl, 0.05 mg/mL Calcofluor white (CFW), and 1 M sorbitol or water (mock) for 24 h. **b** Growth inhibition rate of WT and mutants on PDA supplemented with various chemicals. Different letters on top of bars represent statistically significant differences between genotypes in the same treatment at $P < 0.05$. Data shown are means ± standard deviation (SD), ($n = 4$)

Cys88), which correspond to two active sites (Cys36 and Cys 82) of yeast ScHYR1 (Fig. 1b), are redox-sensitive and essential for H_2O_2 detoxification (Delaunay et al. 2002; Zhang et al. 2008). A similar finding is observed in *M. oryzae* (Huang et al. 2011a) and *A. alternata* (Yang et al. 2016). Moreover, the predicted 3D structure of LtGPX3 shared a robust similarity with chain A of the crystal structure of ScHYR1 (Fig. 1c) (Zhang et al. 2008). These results suggest that the LtGPX3 protein is the structural homolog of ScHYR1 and is highly conserved across different filamentous fungi.

LtGPX3 modulates stress response of *L. theobromae*

Fungal phytopathogens must cope with different environmental stresses to maximize their fitness during life cycles (Singh et al. 2021). The inhibition of the growth rate was used to assess the effect of *LtGPX3* on the response of *L. theobromae* to environmental stresses. Growth assays indicated that deletion of *LtGPX3* resulted in severe sensitivity to KCl, suggesting that *LtGPX3* is required for high salt stress tolerance. In contrast, *ΔLtgpx3* mutants showed decreased sensitivity to CFW- and sorbitol-induced stresses, indicating that *LtGPX3* negatively affects cell wall and osmotic stress responses in *L. theobromae* (Fig. 3). Likewise, in *A. alternata*, *ΔAagpx3* mutants grow faster than the WT on CFW-containing medium (Yang et al. 2016).

Fungal pathogens initiate intricate strategies for ROS neutralization and protection from ROS-mediated damage to counteract plant-derived oxidative burst during progression (Yaakoub et al. 2022). Under oxidative stress, the inhibition of the growth rate of the *ΔLtgpx3* mutants was remarkably higher than that of the WT (Fig. 4a, b), suggesting that *LtGPX3* is required for resistance to ROS-generating oxidants. In *A. alternata*, *AaGPX3* is also required for cellular resistance to various ROS-producing oxidants (Yang et al. 2016). Furthermore, the growth of fungal strains deficient in *GPX3/HYR1* ortholog is highly inhibited by H_2O_2 in *M. oryzae, Hypsizygus marmoreus,* and *Ganoderma lucidum*, suggesting that GPX3/HYR1 orthologs in different fungi have well-conserved roles in fungal adaptation to oxidative stress (Huang et al. 2011a; Li et al. 2015; Zhang et al. 2020). Moreover, the expression of *LtGPX3* was induced under H_2O_2 or TBHP exposure (Fig. 4c, d), similar to those observed in *A. alternata* (Yang et al. 2016) and *G. lucidum* (Li et al. 2015), indicating an essential role of *LtGPX3* in the fungal oxidative stress response. However, the molecular mechanism by which *LtGPX3* regulates the oxidative response requires future investigation.

LtGPX3 is essential for fungal virulence and plant defense suppression

Glutathione peroxidases have been identified to be associated with the virulence of several fungal pathogens, including *M. oryzae* (Huang et al. 2011a), *A. alternata* (Yang et al. 2016), and *V. mali* (Feng et al. 2021). *LtGPX3* was shown to be required for fungal virulence due to reduced lesions in the deletion mutant-inoculated shoots (Fig. 4). One piece of evidence for the role of *LtGPX3* in virulence may be related to its function in ROS detoxification. During *L. theobromae* infection, large amounts of ROS are produced from peach hosts, and the expression of *LtGPX3* is highly induced (Zhang et al. 2020). The ability of *L. theobromae* to detoxify ROS is critical for virulence in peach plants (Zhang et al. 2021). In this study, *ΔLtgpx3* mutants showed increased sensitivity to oxidative stress (Fig. 4a, b), and the mutant-inoculated shoots exhibited increased ROS accumulation at the inoculation site (Fig. 6a, b). Moreover, when an NADPH oxidase inhibitor disrupted plant ROS generation, *ΔLtgpx3*

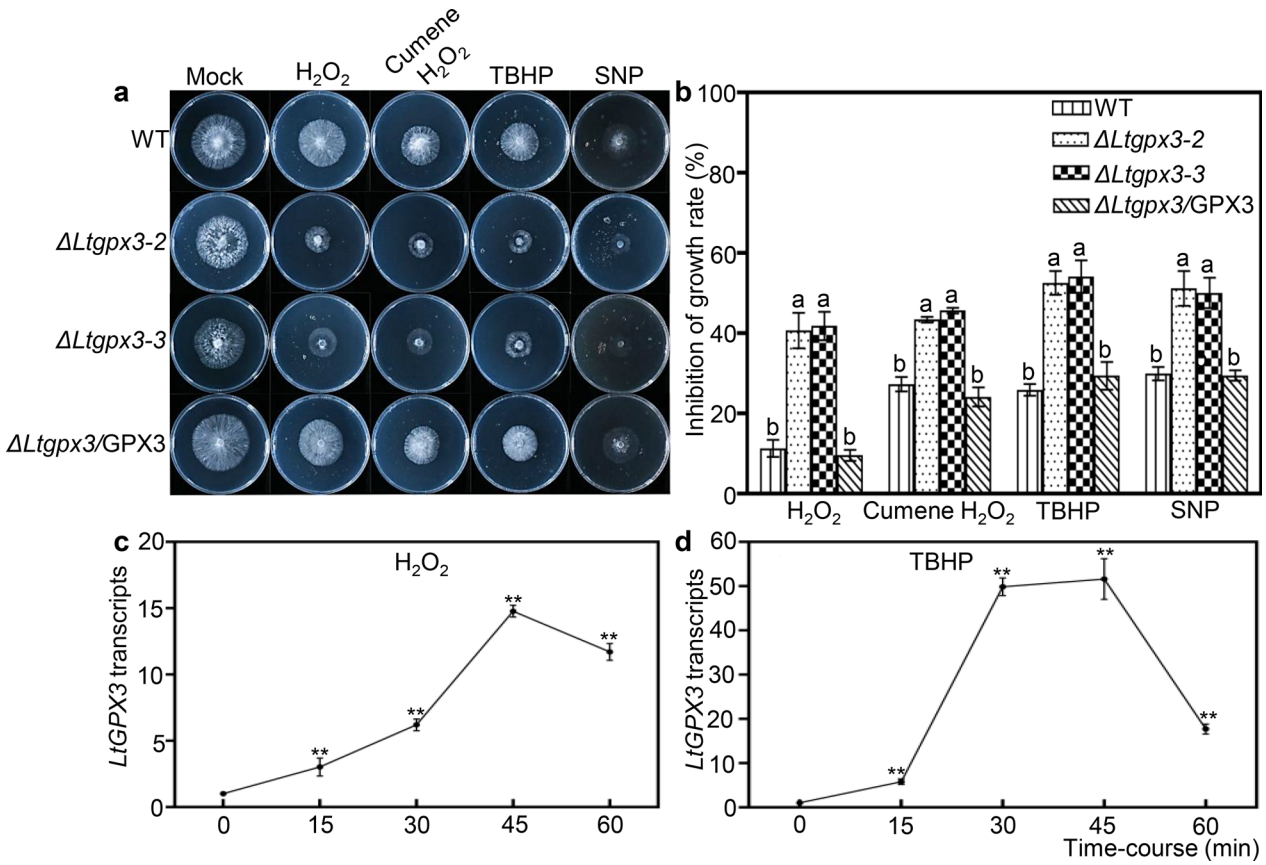

Fig. 4 *LtGPX3* is required for resistance to oxidative stress. **a** Mycelial morphology of WT, two deletion mutants, and the complementation strain *ΔLtgpx3/*GPX3, cultured on PDA media supplemented with 5 mM H_2O_2, 0.68 mM cumene H_2O_2, 0.5 mM TBHP (*tert*-butyl-hydroperoxide), 5 mM SNP (sodium nitroferricyanide dihydrate), or water (mock) for 24 h. **b** Inhibition rate of fungal growth on PDA with oxidants compared to the mock. Letters on top of bars represent statistically significant differences at $P < 0.05$. Data are means ± SD of three biological replicates. **c** and **d** Time-course response of *LtGPX3* transcripts to H_2O_2 or TBHP exposure. Transcript levels were normalized with reference gene *LtTUB* and are displayed relative to the transcript level in samples at time 0 (which was therefore set to 1). Asterisks indicate a significant difference from the initial point (0 min) at $P < 0.01$. Data are means ± SD of three biological replicates

mutant-inoculated shoots showed increased gum release and necrotic lesions (Fig. 7). These results support that *LtGPX3*-mediated ROS detoxification is essential for *L. theobromae* virulence in peach shoots, and similar effects of *GPX3* orthologs on virulence are found in *M. oryzae*, *A. alternata*, and *V. mali* (Feng et al. 2021; Huang et al. 2011a, b; Yang et al. 2016). These findings suggest that fungal *GPX* orthologs may have highly conserved functions in virulence directly related to their role in ROS detoxification.

On the other hand, plant defense responses also contribute to restrict pathogen proliferation and spreading around the infection site. During phytopathogen attack, plant-generated ROS as signaling molecules trigger defense responses, such as the upregulation of *PR* gene transcription (Singh et al. 2021). Our results showed that ROS levels were dramatically increased in the *ΔLtgpx3* mutant-inoculated shoots (Fig. 6). Simultaneously, transcript levels of multiple genes involved in the SA pathway and defense responses were highly induced in the *ΔLtgpx3* mutant-inoculated shoots (Fig. 8). These results demonstrate that *LtGPX3* is involved in ROS signaling-mediated plant defense responses.

Previous studies have shown that the ability to suppress plant defense responses contributes to fungal virulence (Singh et al. 2021). For example, transcription factor MoATF1 functions in the virulence of *M. oryzae* by impairing ROS-mediated plant defense, in which the transcript levels of plant defense-related genes are substantially upregulated in the *Moatf1* mutant-infected rice; similar findings have been observed for the *MoTRX2* mutant of *M. oryzae* (Guo et al. 2010; Wang et al. 2017). In addition, the host rice plants infected with the *ΔModes1* (a host-defense suppressor virulence gene) mutant show strong defense responses with ROS bursts and increased expression of defense-related genes (Chi

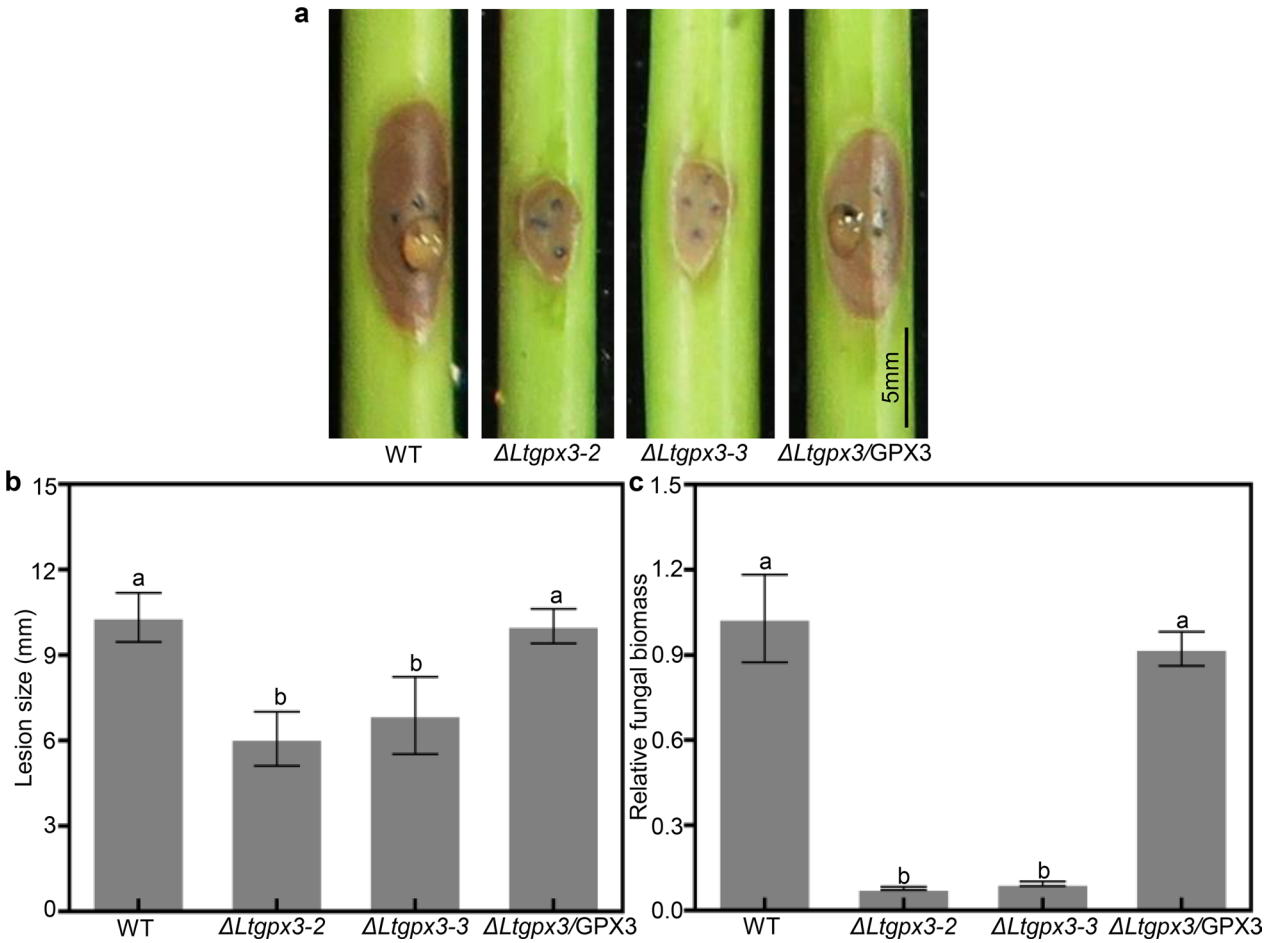

Fig. 5 Virulence test of *LtGPX3* mutants in peach shoots. **a** Peach gummosis development in detached current-year shoots inoculated with different genotypes of *L. theobromae* (WT, deletion mutants, and the complementation strain *ΔLtgpx3*/GPX3) at 5 dpi. **b** Quantification of lesion size on inoculated peach shoots at 5 dpi. **c** qRT-PCR analysis of *L. theobromae* biomass in the infected peach shoots. Different letters on top of bars indicate statistically significant differences at $P < 0.05$

et al. 2009). Deletion of a *MoSOD* gene involved in neutralizing plant-derived ROS disturbs rice defense suppression (Fernandez et al. 2014). These findings highlight the crucial role of suppressing ROS-mediated plant defenses in fungal pathogenesis. Our results show that the reduced virulence of the *LtGPX3* mutant is partly due to the impaired suppression of ROS-mediated peach defense responses. Thus, we have uncovered the function of a GPX3 ortholog in plant defense suppression in filamentous fungi.

Conclusions

We characterized *LtGPX3*, a glutathione peroxidase gene resembling yeast *GPX3/HYR*1-like. Deletion of *LtGPX3* from the *L. theobromae* genome heightened the fungal sensitivity to oxidative stress, resulting in reduced lesions and fungal biomass in the infected shoots. Our findings further revealed the enhanced accumulation of ROS and transcripts of several plant defense-related genes in the *ΔLtgpx3* mutant-inoculated peach shoots, underscoring the crucial roles of *LtGPX3* in ROS detoxification and suppression of plant defense. In addition, we observed that inhibiting plant NADPH oxidase partially restored the virulence of the mutants. These results strongly support a pivotal role for ROS detoxification during *L. theobromae* pathogenesis in peach and establish LtGPX3 as a crucial virulence factor necessary for the fungal pathogen to detoxify ROS generated by host oxidative burst and suppress plant defense responses. In conclusion, our studies provide new insights into the strategy for peach gummosis management by modifying the fungal ROS-detoxifying system.

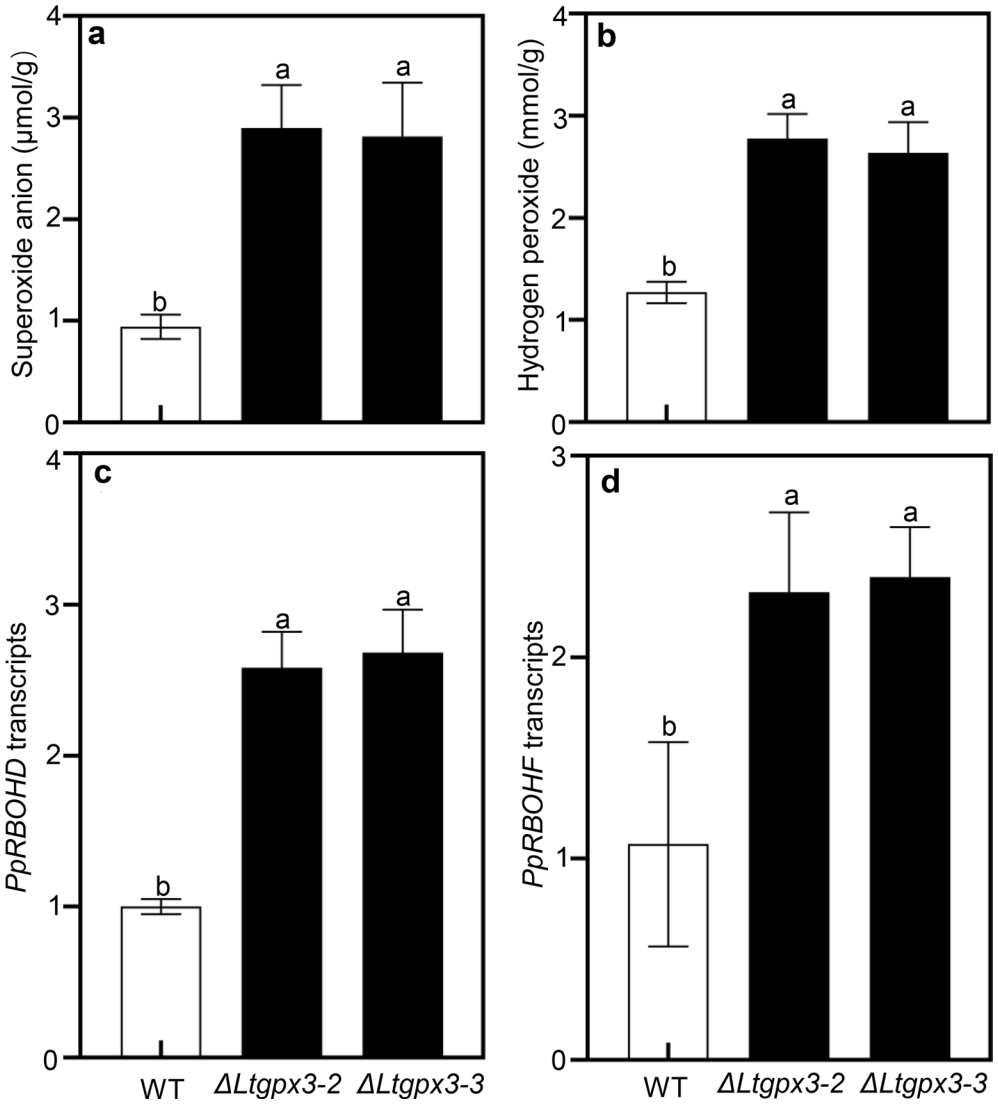

Fig. 6 Effect of *LtGPX3* deficiency on ROS generation and its related gene transcript levels in inoculated peach shoots. **a** and **b** Accumulation of superoxide anion and hydrogen peroxide in peach shoots inoculated with *L. theobromae* WT or *ΔLtgpx3* mutants at 5 dpi. **c** and **d** Transcript abundance of ROS production-related genes *PpRBOHD* and *PpRBOHF* in inoculated peach shoots at 5 dpi. Relative transcript levels of genes compared with WT using reference gene *PpTEF2* for normalization. Data are means ± SD of three biological replicates. In panels, letters on top of bars indicate statistically significant differences at $P < 0.05$

Methods

Fungal strains, culture conditions, and chemical treatments

Wild-type *L. theobromae* isolate JMB122 (Wang et al. 2011) and its genetic derivatives were used as inocula and cultured on PDA (Solarbio, China) at 28°C with a 12 h light/dark photoperiod regime.

To examine the effects of chemical treatment on hyphal growth, WT strain and transformants were grown on PDA plates supplemented with chemical reagents. The integrity of cell walls was tested on PDA medium supplemented with 0.05 mg/mL CFW. PDA supplemented with 1 M sorbitol or 1 M KCl was used to simulate osmotic and salt stress treatments, respectively. As to oxidative stress, PDA was supplemented with 5 mM H_2O_2, 0.5 mM TBHP, 0.68 mM cumene H_2O_2, or 5 mM SNP. PDA without amendment was used as control. Colony diameters were recorded after 24 h of incubation using a digital caliper. The inhibition of growth rate (%) was calculated as described by Zhang et al. (2021). To artificially block plant-derived ROS generation, 0.4 μM DPI (an NADPH oxidase inhibitor) was evenly sprayed onto peach shoots at 12 and 24 h after inoculation with *ΔLtgpx3* mutants. Each experiment was repeated three times with four technical replicates.

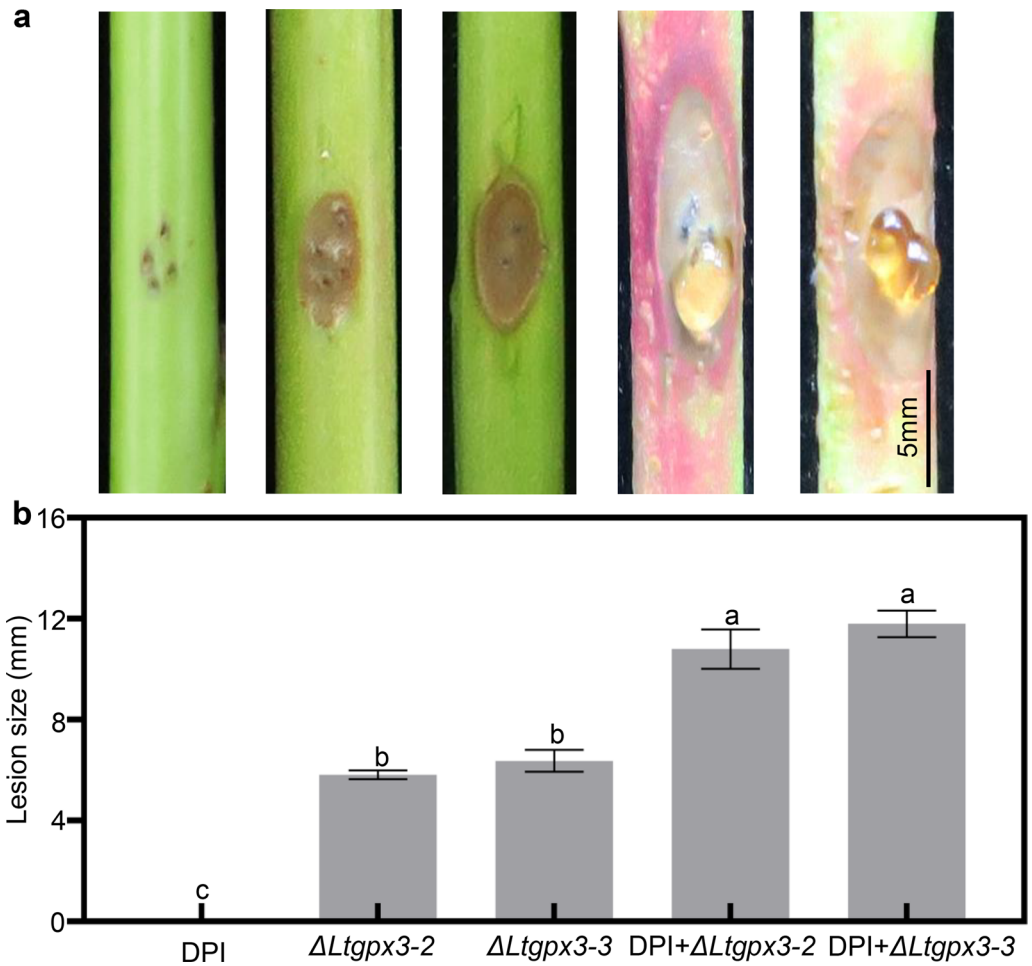

Fig. 7 Impact of diphenylene iodonium (DPI) on the virulence of the *ΔLtgpx3* mutants. **a** Symptom of *ΔLtgpx3* mutants-inoculated peach shoots treated with 0.4 μM DPI (an NADPH oxidase inhibitor) or not at 5 dpi. **b** Quantification of lesion size in infected shoots as shown in **a**. Data are means ± SD of three biological replicates. In panels, letters on top of bars indicate statistically significant differences at $P < 0.05$

Quantitative reverse-transcription PCR (qRT-PCR)

Total RNA extraction, cDNA synthesis, quality and quantity analysis, and qRT-PCR were performed as described previously (Zhang et al. 2022; Zhang et al. 2020). Gene-specific primers for qRT-PCR were designed with the Primer-BLAST program and synthesized by Sangon Biotech (China). Primer sequences are presented in Additional file 2: Table S1. The relative transcripts of genes were normalized with *translation elongation factor 2* (*PpTEF2*) of *P. persica* and *tubulin* (*LtTUB*) of *L. theobromae*, and calculated with the comparative $2^{-\Delta\Delta CT}$ method (Zhang et al. 2020).

Gene cloning and identification

A pair of primers, FD120 and FD121, were used to amplify *LtGPX3* using genomic DNA and cDNA samples of JMB122 (Meng et al. 2022). The amino acid sequence of LtGPX3 was analyzed using the PROSITE program. The orthologs from different species were retrieved using LtGPX3 as a BLASTp query. Protein sequences of LtGPX3 and its orthologs were aligned using ClustalX2 (Larkin et al. 2007), and then a phylogenetic tree was built with MEGA 6.0 using the neighbour-joining method (Tamura et al. 2013). Three-dimensional (3D) structural models of GPX3 proteins were produced by Phyre2 (Kelley and Sternberg 2009) and visualized under PyMOL 2.3.

Gene disruption and complementation

LtGPX3 mutation was conducted via homologous recombination and CRISPR/Cas9 approach (Zhang et al. 2021). Split marker fragments overlapping within the hygromycin phosphotransferase gene (*HYG*) cassette and *LtGPX3* were employed in the PCR strategy (Fig. 2a). The relevant primers are shown in Additional file 2: Table S1. The upstream (2501 bp) and downstream (2697 bp)

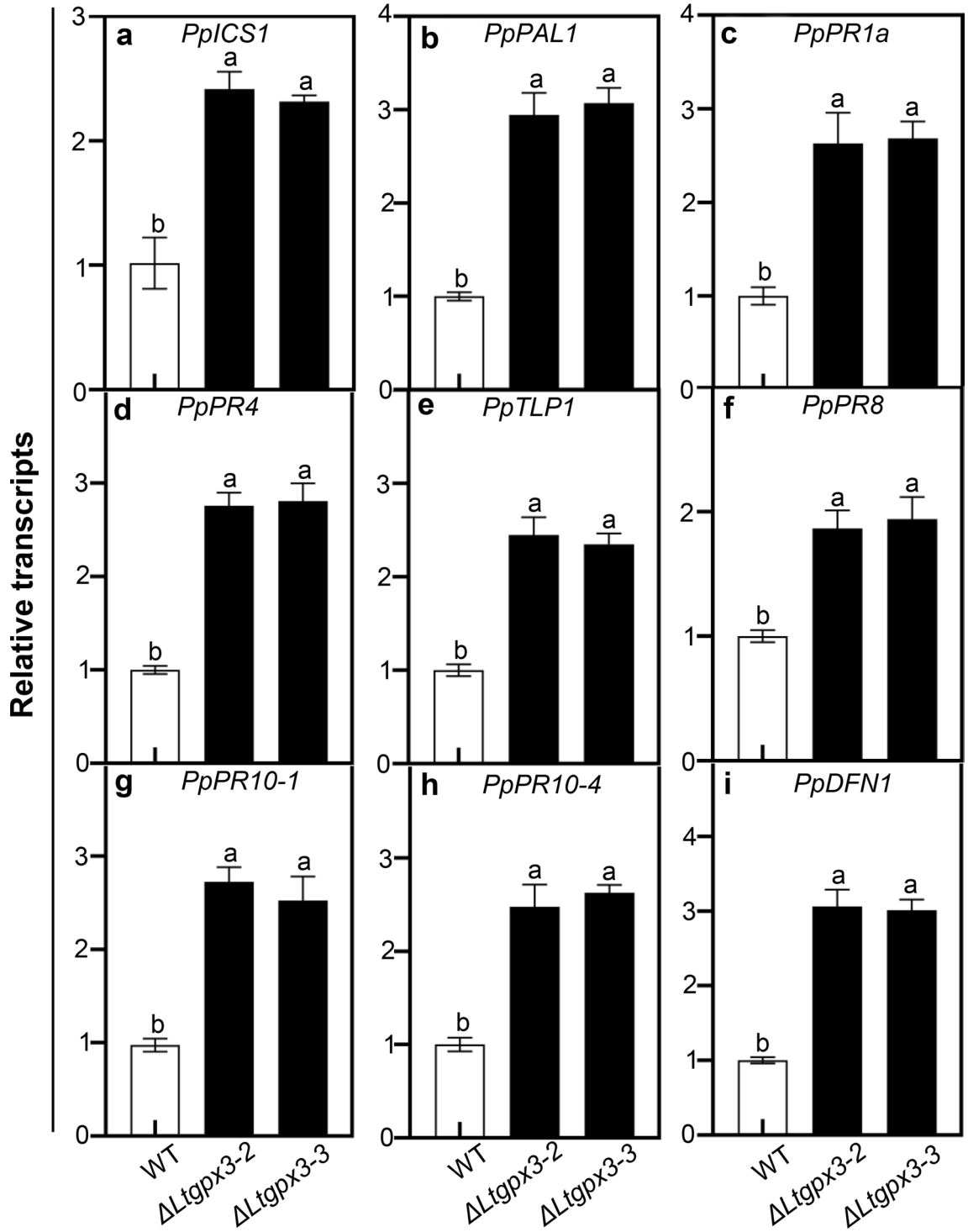

Fig. 8 Impact of *LtGPX3* on transcript levels of plant defense-responsive genes in infected peach shoots. RNA samples were collected from peach shoots inoculated with WT or *ΔLtgpx3* mutants at 5 dpi. Relative transcript levels of tested genes are compared with the control (WT) using reference gene *PpTEF2* for normalization. Data are means ± SD of three biological replicates. In panels, letters on top of bars indicate statistically significant differences at $P < 0.05$

regions of *LtGPX3* and a fragment of the hygromycin B resistance phosphotransferase gene (*HPH*, 1423 bp) in the pBHt2 vector were separately cloned. Subsequently, two fragments of 5′*LtGPX3*::HY/g and h/YG::3′*LtGPX3* were constructed with the primers as indicated in Fig. 2a. A 20-nucleotide segment before NGG in the exon of *LtGPX3* was selected, and amplified using primers modified with sticky ends (5′ end) for single-guide RNA (sgRNA), which was inserted into the linearized pmCas9 vector (Fig. 2b) to produce the pmCas9-*LtGPX3* plasmid. Eventually, *LtGPX3* deletion mutant (*ΔLtgpx3*) was created by co-transforming the aforementioned two constructs and pmCas9-*LtGPX3* into JMB122 protoplasts.

The full-length coding sequence of *LtGPX3* and its promoter (1500 bp upstream of the ATG start codon but no stop codon) was amplified from the genomic DNA of strain JMB122 using primers 18F/18R and then used to generate the complementation strain *ΔLtgpx3/*GPX3. The *LtGPX3* fragment was fused with a neomycin resistance gene (*NEO*) responsible for G418 resistance driven by the *Aspergillus nidulans trpC* promoter. Subsequently, the *LtGPX3::NEO* plasmid was transformed into protoplasts of *ΔLtgpx3*. The deletion transformants were selected from the regeneration medium (Zhang et al. 2021) supplemented with 150 μg/mL hygromycin B (Roche, Switzerland), and the hygromycin-resistant transformants were PCR-screened using primers 16F/16R and 17F/17R. The complementation transformants showing resistance to G418 were PCR verified using primers 15F/15R.

Virulence assay

Disease tests were conducted on peach shoots as described earlier (Zhang et al. (2020). Necrotic lesions were photographed at 5 dpi. The green bark tissues within 0.5–1.0 cm of the injury site were shortly placed in liquid nitrogen and stored at − 80°C. Relative fungal biomass in infected shoot tissues was analyzed as described previously (Zhang et al. 2021). The assays were independently repeated three times, each comprising 15 shoot segments.

Analysis of superoxide anion and hydrogen peroxide

Accumulations of O_2^- and H_2O_2 were measured using reported methods (Shen et al. 2021; Zhang et al. 2020). The absorbances of O_2^- (μmol/g FW) and H_2O_2 (mmol/g FW) were recorded at 530 and 415 nm, respectively.

Statistical analysis

All datasets represent the means ± SD of at least three biological replicates. Different letters show statistically significant differences as evaluated by one-way analysis of variance (ANOVA) using Duncan's multiple range test of SAS (version 8.1, SAS Institute, USA) at $P < 0.05$. Asterisks represent statistical significance from Student's *t*-test at $P < 0.05$ (∗) or 0.01 (∗∗).

Abbreviations

AP1	Activating protein 1
GPX	Glutathione peroxidase
H_2O_2	Hydrogen peroxide
HYG	Hygromycin phosphotransferase gene
ICS1	Isochorismate synthase 1
LTP1	Lipid-transfer protein 1
O_2^-	Superoxide anion
PAL1	Phenylalanine ammonia lyase 1
PR	Pathogenesis-related genes
RBOHs	Respiratory burst oxidase homologs
ROS	Reactive oxygen species
SNP	Sodium nitroferricyanide dihydrate
TEF2	Translation elongation factor 2
YAP1	Yeast-activating protein 1

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s42483-024-00224-1.

Additional file 1: Figure S1. Phylogenetic analysis of GPX3 proteins. **Figure S2.** Mycelial morphology of WT and *ΔLtgpx3* transformants cultured on PDA media supplemented with 150 μg/mL hygromycin B or water (mock) for 24 hours. **Figure S3.** Relative expression of the *LtGPX3* gene in WT and *ΔLtgpx3* transformants.

Additional file 2: Table S1. Primer sequences used in this study.

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Author contributions

HZ, GL, and JL designed the experiments; HZ performed all the experiments with occasional help from XS, WS, DZ, and XH; HZ and WS analyzed the data; HZ, KZ, and JL wrote the manuscript. All authors have read and approved the final manuscript.

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Availability of data and materials

The datasets used and analyzed during the current study are available from the corresponding author upon reasonable request.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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