A functional analysis of mitochondrial respiratory chain cytochrome $bc_1$ complex in *Gaeumannomyces tritici* by RNA silencing as a possible target of carabrone

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Abstract

*Gaeumannomyces tritici*, an ascomycete soilborne fungus, causes a devastating root disease in wheat. Carabrone, a botanical bicyclic sesquiterpenic lactone, is a promising fungicidal agent that can effectively control *G. tritici*. However, the mechanism of action of carabrone against *G. tritici* remains largely unclear. Here, we used immunogold for subcellular localization of carabrone and the results showed that carabrone is subcellularly localized in the mitochondria of *G. tritici*. We then explored the functional analysis of genes $GtCytc_1$, $GtCytb$, and $GtIsp$ of the mitochondrial respiratory chain cytochrome $bc_1$ complex in *G. tritici* by RNA silencing as a possible target of carabrone. The results showed that the silenced mutant $\Delta GtIsp$ is less sensitive to carabrone compared to $\Delta GtCytc_1$ and $\Delta GtCytb$. Compared with the control, the activities of complex III in all the strains, except $\Delta GtIsp$ and carabrone-resistant isolate 24-HN-1, were significantly decreased following treatment with carabrone at $EC_{20}$ and $EC_{80}$ in vitro (40%–50% and 70%–80%, respectively). The activities of mitochondrial respiratory chain complex III and the mitochondrial respiration oxygen consumption rates in all the strains, except $\Delta GtIsp$ and 24-HN-1, were higher with respect to the control when treated with carabrone at $EC_{20}$ in vivo. The rates of mitochondrial respiration of all strains, except $\Delta GtIsp$, were significantly inhibited following treatment with carabrone at $EC_{80}$ (ranging from 57% to 81%). This study reveals that the targeting of the iron–sulphur protein encoded by $GtIsp$ is highly sensitive to carabrone and provides a direction for the research of carabrone’s target.

**KEYWORDS**
carabrone, cytochrome $bc_1$, *Gaeumannomyces tritici*, immunogold, mitochondrial respiratory chain, RNA silencing
**INTRODUCTION**

*Gaeumannomyces tritici*, an ascomycete soilborne fungus, causes a devastating root disease of wheat called take-all. The fungus was previously named *Gaeumannomyces graminis var. tritici*, and recently Hernandez-Restrepo et al. recombined its name as *Gaeumannomyces tritici* (Hernandez-Restrepo et al., 2016). Take-all is the most devastating wheat root disease in all cultivation areas worldwide (Cook, 2003). It also causes damage to other cereal crops, including rye, triticale, and barley, but to a lesser extent (Walker, 1980; Ward and Bateman, 1999; Freeman and Ward, 2004). Black necrosis on the roots is a typical symptom of infection by *G. tritici* mycelia. The stunting and premature ripening of crops, which results in shrivelled grain, has the capacity to cause significant yield declines. These decreases in yield and quality have resulted in substantial financial losses (Freeman and Ward, 2004). However, the effective and economic control of the disease remains difficult. As fungicides are restrictive (Freeman and Ward, 2004). However, the effective and economic control of the disease remains difficult. As fungicides are restrictive and effective only during the seedling phase of take-all, biofungicides represent the best alternative for long-term control. Thus, a new biofungicide that exhibits low toxicity and confers few negative effects on the environment is urgently required to control take-all.

Carabrone is a bicyclic sesquiterpenic lactone isolated from the powdered fruits of *Carpesium macrocephalum*. These plants are widely distributed in Asia and Europe, especially in the mountainous areas of south-west China (Minato et al., 1964; Zhang et al., 2016). The extraction of carabrone has been described previously by Minato et al. (1964). Carabrone displays antibacterial and antitumour activities, and is associated with low toxicity and few negative effects on environmental health (Feng et al., 2010; Wang et al., 2014).

In our previous study, we reported that carabrone exerts significant antifungal activity against *G. tritici* (Wang et al., 2017b, 2017c). Using transmission electron microscopy, carabrone was found to have a significant effect on biological oxidation and caused mitochondrial vacuolation in *G. tritici*. Subsequently, we identified the mitochondria as the subcellular location of carabrone by confocal microscopic detection of a fluorescently labelled molecules (Wang et al., 2017b). We further reported that the mitochondrial respiratory chain complex III in *G. tritici* is highly sensitive to carabrone and is a potential target of carabrone (Wang et al., 2017c).

The mitochondrial respiratory chain cytochrome bc₁ complex (complex III, ubiquinolcytochrome c oxidoreductase) is a central segment in the mitochondrial respiratory chain. Mitochondrial complex III catalyses electron transfer from ubiquihydroquinone to cytochrome c, accompanied by vectorial proton translocation across the membrane, thus generating ATP (Kim and Zitomer, 1990; Lange and Hunte, 2002). The mitochondrial cytochrome bc₁ complex isolated from beef heart is a dimer, with each monomer comprising 11 different polypeptide subunits, and the relative molecular mass of a monomer is 240 kDa (Iwata et al., 1998). Every cytochrome bc₁ complex contains three common subunits with active redox centres, namely, cytochrome b, cytochrome c₁, and an iron–sulphur cluster of the Rieske protein. The two haem groups of cytochrome b (b₅₅ and b₅₆) form an electrical circuit across the mitochondrial membrane, and an applied membrane potential moves an electron from one haem to another (Trumpower, 1990). Electron transfer from ubiquihydroquinone to cytochrome c occurs through the Q-cycle mechanism within the cytochrome bc₁ complex, consisting of a separate ubiquinone-reducing site (Qₒ) and a ubiquinol-oxidizing site (Qₚ). Antimycin is thought to bind at the Qₚ site postulated in the Q-cycle mechanism (Gao et al., 2002). The fungicidal activity of strobilurins stems from their ability to inhibit the cytochrome bc₁ complex by binding at the Qₒ site of cytochrome b (Gisi et al., 2002). The Qₚ and Qₒ inhibitor-binding sites, and especially the Qₒ site, are the targets of drug-design efforts to produce effective and environmentally safe plant-protection fungicides (Zhang et al., 1998; Bartlett et al., 2002).

RNA interference or RNA silencing (RNAi) is a conserved mechanism through which double-stranded RNA (dsRNA) molecules induce the inactivation of homologous sequences by mRNA translation inhibition, chromatin remodelling, degradation, or DNA elimination (Nunes and Dean, 2012; Carreras-Villaseñor et al., 2013). RNA silencing pathways are associated with small RNAs (20–30 nucleotides) that function as specificity factors for inactivating homologous sequences via a variety of mechanisms. At least three classes of small RNAs have been identified thus far, namely, short interfering RNAs (siRNAs), microRNAs (miRNAs), and piwi-interacting RNAs (piRNAs). They are contained in the RNA-induced silencing complex (RISC) or a nuclear form of RISC called the RNA-induced transcriptional silencing complex (RITs; Moazed, 2009). The expression of hairpin RNAi or antisense constructs or other forms of siRNA molecules directed against the target transcripts of pests or diseases has been used to control both insects and diseases in plants (Cheng et al., 2015). The RNAi-related phenomenon was first described in an ascomycete, and it has been shown that the RNAi pathway participates in various developmental and physiological processes, including gene regulation, genomic stability, invasive nucleic acid defence, heterochromatin formation, and gene regulation in several fungal species (Chang et al., 2012; Nicolás et al., 2013; Chen et al., 2015). The use of RNAi in *Fusarium graminearum* is relatively mature, and it has been established that the Dicer protein Fgdicer2 and Argonaute protein Fgago1 play important roles in the silencing process (Cheng et al., 2015). Before this study, RNAi has not been used in *G. tritici*.

By means of immunogold subcellular localization, we previously demonstrated that carabrone is subcellularly localized in the mitochondria of *G. tritici*. In the current study, we thus investigated the potential and mechanism of action of carabrone on mitochondrial respiratory chain complex III in *G. tritici*. The genes *GtcYctc*, *GtcYtb*, and *Gtisp* were selected from the catalytic core of the mitochondrial respiratory chain complex III. We analysed the effect of carabrone on these genes of the cytochrome bc₁ complex by RNAi and overexpression. The silencing and overexpression mutants were successfully obtained of the genes *GtcYctc*, *GtcYtb*, and *Gtisp* in *G. tritici*. In addition, we tested the mycelial growth rates, mycelial morphology, melanin, enzyme activity, pathogenicity, and mitochondrial respiration rates in wild-type strain and mutants of *G. tritici*. We conclude that the iron–sulphur protein encoded by the gene *Gtisp* has the possibility of being a potential target of carabrone.
2 | RESULTS

2.1 | Immunogold subcellular localization

Based on our previous research, the spleen cells from a mouse that was immunized with a high dose of carabrone were used for the fusion experiment (Wang et al., 2017b). Supernatants from the 96-well plates were first assessed by noncompetitive enzyme-linked immunoassay (ELISA). The hybridoma was further verified by indirect competitive ELISA using carabrone as a competitor. A total of seven clones of the positive hybridoma gave positive results, with specific binding to free carabrone. Out of the seven clones, two stable clones were selected for subcloning for three cycles by limiting the dilution to one cell per well. Finally, one stable hybridoma clone, F2B4, which exhibited the broadest activity to carabrone, was selected to produce monoclonal antibodies. The optimum indirect competitive (ic)-ELISA conditions were determined to be a 1 μg/ml coating antigen (TOVA) concentration and a 1:16 x 10^5 ratio of the antibody (F2B4) dilution. As shown in Table S1, the IC_{50} values of the carabrone, carboral, and γ-phenyl-α-methylene-γ-butylactone were 2.05 ng/L, 112.96 ng/L, and 4,169.06 ng/L, respectively. According to the formula, the cross-reactivity values of carabrone and γ-phenyl-α-methylene-γ-butylactone were 1.8 and 0.049, respectively (Table S1).

Following the incubation of ultrathin sections of G. tritici with the monoclonal antibody and secondary antibody, the mitochondria of G. tritici were intensely and regularly labelled with gold particles. There were no gold particles in G. tritici that was incubated without carabrone (EC_{50}; Figure 1a–c). The wall and inner membranes of the cell showed a high density of gold particles when incubated with carabrone for 30 min (Figure 1d). There were numerous particles in both the cell membranes and the mitochondria when incubated with carabrone for 1 hr (Figure 1e). With the increase in carabrone incubation time for 2, 6, 12, and 48 hr, no gold particles were observed in the other organelles, and the labelling density increased towards the mitochondria (Figure 1f–i). Interestingly, the mitochondrial ridges appeared blurred when incubated with carabrone for over 6 hr and the mitochondria were vacuolar when incubated with carabrone for 48 hr.

2.2 | Quantitative reverse transcription PCR

We analysed the mRNA expression and DNA levels of the genes GtCytb1, GtCytb, and GtIsp in the wild-type strain ACC30310, carabrone-resistant isolate 24-HN-1, silencing mutants ΔGtCytb1, ΔGtCytb, ΔGtIsp, and overexpression mutants OECytb, OECytb, and OEIs in growth rates on potato dextrose agar (PDA; Figure 2e). Carabrone sensitivity tests showed that the wild-type progenitor and overexpression mutants OECytb, OECytb, and OEIs were highly sensitive to carabrone (100 μg/ml). The sensitivity of ΔGtCytb1, ΔGtCytb, ΔGtIsp, and 24-HN-1 to carabrone was relatively low compared with the wild-type, and the silenced mutant ΔGtIsp was less sensitive to carabrone with respect to ΔGtCytb1 and ΔGtCytb (Table 1 and Figure 3). The superoxide anion, H_2O_2, and the hydroxyl radical are known as reactive oxygen species (ROS). Adding exogenous H_2O_2 can increase the oxidative stress to the strain and measure the strain’s reactivity to oxidative stress. Oxidative stress generated by 10 mM H_2O_2 did not significantly affect the ΔGtCytb1, ΔGtCytb, and ΔGtIsp mutants on the PDA plates. Compared with the control, the mycelial growth of the wild-type progenitor and the overexpression mutants OECytb, OECytb, and OEIs was significantly inhibited by approximately 60%–70% following treatment with 10 mM H_2O_2, while the mycelial growth of 24-HN-1 was inhibited by about 20% compared with the control (Figure 3).

The mycelial morphology of the wild-type progenitor, ΔGtCytb1, ΔGtCytb, ΔGtIsp, 24-HN-1, OECytb1, OECytb, and OEIs treated or untreated with carabrone (100 μg/ml) and H_2O_2 (10 mM) was observed by scanning electron microscopy. Compared with the wild-type progenitor, the hyphae of the ΔGtCytb1, ΔGtCytb, and ΔGtIsp mutants became thinner and had fewer hyphal branches, whereas multiple hyphae coalesced to grow together in 24-HN-1. The hyphae of the wild-type progenitor were intertwined with each other and had fewer hyphal branches following treatment with carabrone or H_2O_2 with respect to the control. Compared with the control, the hyphae
**FIGURE 1** Immunological localization of carabrone in *Gaeumannomyces tritici*. (a) Blank control. (b) There were no gold particles when incubated with the monoclonal antibody and secondary antibody. (c) There were no gold particles when incubated with the secondary antibody. (d) The inner membranes of the cell showed a high density of gold particles when incubated with carabrone (30 min), the monoclonal antibody, and secondary antibody. (e) There were a lot of particles both in the cell membranes and mitochondria when incubated with carabrone (1 hr), the monoclonal antibody, and secondary antibody; the mitochondria exhibited a strong labelling with gold particles when incubated with carabrone for 2 hr (f), 6 hr (g), 12 hr (h), and 48 hr (i), and then incubated with the monoclonal antibody and secondary antibody. Arrows indicate the position of the gold particles. “V” indicates that the mitochondrial ridges appeared blurred when incubated with carabrone over 6 hr. The mitochondria were vacuolar when incubated with carabrone for 48 hr. Bar = 500 nm.

**TABLE 1** List of *Gaeumannomyces tritici* isolates used in this study

| Isolate     | EC<sub>50</sub> (µg/ml)<sup>a</sup> | Complex III mutation<sup>b</sup> | Source or reference |
|-------------|-------------------------------------|-----------------------------------|---------------------|
| ACCC30310   | 40.30                               | -                                 | This study          |
| ΔGtCytc<sub>1</sub> | 104.94                            | Silence (Cytc<sub>1</sub>)        | This study          |
| ΔGtCytb     | 113.19                              | Silence (Cytb)                    | This study          |
| ΔGtIsp      | 156.18                              | Silence (Isp)                     | This study          |
| 24-HN-1     | 182.73                              | Carabrone-resistant isolate       | Zhang (2016)        |
| OECytc<sub>1</sub> | 32.44                              | Overexpression (Cytc<sub>1</sub>) | This study          |
| OECytb      | 30.02                               | Overexpression (Cytb)             | This study          |
| OEIsp       | 25.88                               | Overexpression (ISP)              | This study          |

<sup>a</sup>Effective concentration of carabrone causing 50% inhibition of growth.

<sup>b</sup>Mitochondrial respiratory chain complex III in *G. tritici*. 
The relative mRNA expression levels, the relative DNA levels, and mycelial growth rate of GtCytc₁, GtCytb, and GtIsp in the wild-type strain ACCC30310 and mutants: (a) ACCC30310, ΔGtCytc₁, and OECytc₁, (b) ACCC30310, ΔGtCytb, and OECytb, and (c) ACCC30310, ΔGtIsp, and OEIsp. (d) The relative DNA levels of GtCytc₁, GtCytb, GtIsp, and GtLac in the wild type, ΔGtCytc₁, ΔGtCytb, ΔGtIsp, carabrone-resistant isolate 24-HN-1, OECytc₁, OECytb, and OEIsp. Expression of the 18S rRNA gene was used as the reference. (e) The mycelial growth rate of the wild-type strain ACCC30310, ΔGtCytc₁, ΔGtCytb, ΔGtIsp, 24-HN-1, OECytc₁, OECytb, and OEIsp mutants. Bars represent mean values ± SD of three independent experiments. Different lowercase letters indicate a significant difference (p < .05).

2.4 | Melanin production

Hyphal melanin was measured by its binding to Azure A and increased as biomass accumulated in the PDB cultures of the wild-type strain, ΔGtCytc₁, ΔGtCytb, ΔGtIsp, 24-HN-1, OECytc₁, OECytb, and OEIsp. The hyphae of 24-HN-1 were highly condensed together following exposure to carabrone or \( \text{H}_2\text{O}_2 \). The mycelia of OECytc₁, OECytb, and OEIsp become seriously distorted and broken when treated with carabrone or \( \text{H}_2\text{O}_2 \) (Figure 4).
The 24-HN-1 strain produced significantly more hyphal melanin (approximately 140 \( \mu \)g/mg dry weight [DW] from 5 to 7 days) than others, whereas the wild-type strain produced little melanin (approximately 75 \( \mu \)g/mg DW for 7 days). There were no significant differences in melanin production by mutants \( \Delta \)GtCytc, \( \Delta \)GtCytb, and \( \Delta \)GtIsp (approximately 130 \( \mu \)g/mg DW from 5 to 7 days), whereas the production of melanin was also significantly higher than the wild-type strain. The OECytc, OECytb, and OEIsp mutants produced melanin (approximately 90 \( \mu \)g/mg DW from 5 to 7 days), and did not differ significantly from each other (Figure 5a).
2.5 | Enzyme activity studies

The peroxidase activity in the wild-type strain was approximately 2.6 U/ml. Compared with the wild-type strain, the peroxidase activity in the ∆GtCytc₁, ∆GtCytb, ∆Gtlsp, and 24-HN-1 mutants was significantly increased (71%, 58%, 65%, and 94%, respectively). The activity of extracellular laccase in the wild-type strain was approximately 4.3 U/ml. Compared with the wild-type strain, the extracellular laccase activity in ∆GtCytc₁, ∆GtCytb, ∆Gtlsp, and 24-HN-1 was significantly decreased (approximately 47%, 37%, 40%, and 21%, respectively). There were no significant differences in the activities of peroxidase and extracellular laccase between the overexpression mutants OECytc₁, OECytb, OEIsp, and the wild-type strain (Figure 5b).

The activity of mitochondrial respiratory chain complex III in the wild-type strain was 61 nmol-min⁻¹·mg⁻¹. The activities of mitochondrial respiratory chain complex III in ∆GtCytc₁, ∆GtCytb, ∆Gtlsp, and 24-HN-1 were significantly decreased (approximately 36%, 61%, 75%, and 77%, respectively) with respect to the wild-type strain. Compared with the control, the activities of complex III in all strains were significantly decreased following treatment with carabrone at EC₂₀ and EC₈₀ in vitro (40%–50% and 70%–80%, respectively), except for ∆Gtlsp and 24-HN-1. The activities of complex III in ∆Gtlsp and 24-HN-1 changed little when treated with carabrone at EC₂₀ and
EC$_{80}$ in vitro (Figure 6a). Compared with the control, the activities of complex III in the wild-type strain, ∆GtCytc$_1$, ∆GtCytb, ∆GtIsp, OECytc$_1$, OECytb, and OEIsp were significantly increased following treatment with carabrone at EC$_{20}$ in vivo (approximately 32%, 33%, 38%, 22%, 10%, and 5%, respectively), except for 24-HN-1 and OEIsp. In contrast to the control, complex III activities in the wild-type strain, ∆GtCytc$_1$, ∆GtCytb, ∆GtIsp, 24-HN-1, OECytc$_1$, OECytb, and OEIsp decreased after exposure to carabrone at EC$_{80}$ in vivo (approximately 61%, 50%, 46%, 20%, 11%, 48%, 56%, and 64%, respectively; Figure 6b).

### 2.6 Pathogenicity

In the pathogenicity assays, the wild-type and ∆GtCytc$_1$, ∆GtCytb, ∆GtIsp, OECytc$_1$, OECytb, and OEIsp mutants infected the wheat roots. In addition, all eight strains decreased wheat seed germination, shoot length, and root biomass compared to the uninfected plants. The wild-type strain significantly reduced seedling shoot length, shoot weight, and root length in comparison to the ∆GtCytc$_1$, ∆GtCytb, ∆GtIsp, and 24-HN-1 mutants, though there were no significant differences between the wild-type strain and the overexpression mutants (Table S2). The eight strains also caused discoloration of the roots, leading to black necrotic lesions, especially in the wild-type strain, OECytc$_1$, OECytb, and OEIsp (Figure 7). The results indicated that the percentage root areas with lesions in the wheat seedlings were 46%, 9%, 6%, 6%, 4%, 38%, 21%, and 42% when infected with the wild-type strain, ∆GtCytc$_1$, ∆GtCytb, ∆GtIsp, 24-HN-1, OECytc$_1$, OECytb, and OEIsp, respectively. The wheat seedlings were severely infected with the wild-type strain and the overexpression mutants. Thus, the ∆GtCytc$_1$, ∆GtCytb, ∆GtIsp, and 24-HN-1 mutants demonstrated weaker virulence to wheat than the wild-type strain and the overexpression mutants (Table S2).

### 2.7 Rates of mitochondrial respiration

The rates of mitochondrial respiration of the wild-type strain, ∆GtCytc$_1$, ∆GtCytb, ∆GtIsp, 24-HN-1, OECytc$_1$, OECytb, and OEIsp with carabrone (control, EC$_{20}$, EC$_{80}$) and antimycin A (20 µg/ml) are shown in Figure S1. Following treatment with carabrone at EC$_{20}$, the rates of mitochondrial respiration of all the strains were higher to varying degrees with respect to the control. The results indicate that a low concentration of carabrone activates mitochondrial alternative oxidase, which functions as a part of the electron transfer chain (Laties, 1982; Vanlerberghe and Ordog, 2002). The rates of mitochondrial respiration of the wild-type strain, ∆GtCytc$_1$, ∆GtCytb, ∆GtIsp, 24-HN-1, OECytc$_1$, OECytb, and OEIsp were significantly inhibited following treatment with carabrone at EC$_{80}$ (approximately 81%, 77%, 79%, 57%, 72%, 73%, and 72%, respectively), while ∆GtIsp was not significantly inhibited. Antimycin A is thought to bind at the Q$_i$ site of cytochrome b in mitochondrial respiratory chain complex III (Gao et al., 2002; Kotiaho et al., 2008), therefore we used antimycin A as the positive control in our evaluation of the effects of carabrone. Following treatment with antimycin A (20 µg/ml), the rates of mitochondrial respiration of the wild-type strain, ∆GtCytc$_1$, ∆GtCytb, 24-HN-1, OECytc$_1$, OECytb, and OEIsp, but not ∆GtIsp, were significantly inhibited (approximately 95%, 90%, 92%, 57%, 92%, 93%, and 90%, respectively).

**FIGURE 5** The determination of melanin and activity of extracellular peroxidase and laccase. (a) The determination of melanin in the wild-type strain *Gaeumannomyces tritici* ACCC30310, ∆GtCytc$_1$, ∆GtCytb, ∆GtIsp, carabrone-resistant isolate 24-HN-1, OECytc$_1$, OECytb, and OEIsp mutants. The strains were cultured in potato dextrose broth (PDB). Melanin was measured by Azure A binding to the hyphae. (b) Extracellular peroxidase and laccase activity of the wild-type strain, ∆GtCytc$_1$, ∆GtCytb, ∆GtIsp, 24-HN-1, OECytc$_1$, OECytb, and OEIsp were measured by an ABTS oxidizing test with or without H$_2$O$_2$ supplementation in cultures grown in PDB. Error bars represent the SD and each point is the average of triplicate cultures. In the same enzyme activity, different lowercase letters indicate a significant difference ($p < .05$).
3 | DISCUSSION

In this study the results of the immunogold subcellular localization demonstrate that carabrone is subcellularly localized in the mitochondria of *G. tritici* (Figure 1), which is consistent with the results obtained by Wang *et al.* (2017b). The preliminary results identified that the specific target proteins of carabrone are localized in the mitochondria. Our previous study showed that carabrone has inhibitory impacts on the respiratory chain, and the mitochondrial respiratory chain complex III in *G. tritici* is highly sensitive to carabrone and is a potential target of this botanical fungicidal agent (Wang *et al.*, 2017c). We show that the delivery of a hairpin RNAi construct targeting the three genes of the mitochondrial respiratory chain cytochrome *bc*₁ complex in the necrotrophic ascomycete fungus *G. tritici* via an hpRNA-expressing plasmid effectively decreases the expression of target genes. Thus, our work has further elucidated the target of carabrone on the mitochondrial respiratory chain complex III in *G. tritici*.

The use of RNAi on *G. tritici* was motivated by the fact that wheat take-all causes serious losses in agricultural yields worldwide, as well as safety issues relating to the use of fungicides to control take-all (Yun *et al.*, 2012). Focusing on mitochondrial respiratory chain complex III genes *GtCytC*, *GtCytb*, and *GtIsp* in *G. tritici* as targets for silencing was reasonable, as these genes are the functional subunits...
(core proteins) of the mitochondrial respiratory chain complex III (LIN et al., 1982). Furthermore, our previous work provided proof-of-concept that the mitochondrial respiratory chain complex III is a potential target of carabrone in G. tritici (Wang et al., 2017c).

Our results also corroborate the finding that silencing of the mutants containing the RNA silencing vector pSilent-1 results in significantly decreased expression levels (ranging from 91% to 99%) of the target mRNAs in G. tritici (Nakayashiki et al., 2005). In addition, the genes GtCytc_1, GtCytb, and GtIsp of the overexpression mutants were significantly up-regulated (ranging from 6 to 9.1 times) in comparison to the wild-type strain (Figure 2a–c). The amount of DNA of the genes GtCytc_1, GtCytb, GtIsp, and GtLac in all the strains changed little, except for 24-HN-1 (Figure 2d), which showed there were no significant differences between the wild-type strain and the silencing and overexpression mutants in the level of genomic DNA. RNAi can effectively reduce the mRNA expression of the target gene in the corresponding silenced mutant strain, but the DNA level does not significantly change, which is consistent with previous studies (Nakayashiki et al., 2005). The results show that the expression of genes GtCytc_1, GtCytb, and GtIsp were successfully altered in G. tritici by silencing and overexpression.

Blocking of the electron transfer in the respiration pathway leads to energy deficiency due to a lack of ATP, which interferes with the expression of the GtCytc_1, GtCytb, and GtIsp genes (Moghaddas et al., 2008). Consistent with these findings, the mycelial growth rates of ΔGtCytc_1, ΔGtCytb, and ΔGtIsp were severely inhibited (25%) compared with the wild-type strain (Figure 2e).

In addition, the hyphae were thin with reduced hyphal branching (Figure 4). The overexpression mutants OECytc_1, OECytb, and OEIsp exhibited increased sensitivity to H_2O_2 with respect to the silencing transformants ΔGtCytc_1, ΔGtCytb, and ΔGtIsp, which indicates the inefficiency of the mitochondrial respiratory chain when silencing the genes GtCytc_1, GtCytb, and GtIsp (Figures 3 and 4). This is also related to the activity of peroxidase. Fungal melanins are dark, polymeric pigments that may offer protection against environmental stresses (Bell and Wheeler, 1986; Frederick et al., 1999). The results showed that 24-HN-1 produced significantly more hyphal melanin than others during growth. The wild-type strain showed the lowest levels of melanin production, and there were not significant differences between silenced mutants ΔGtCytc_1, ΔGtCytb, and ΔGtIsp (Figure 5a). Melanin can protect the hyphae and enhance the ability to infect and colonize. The increase in melanin content of the ΔGtCytc_1, ΔGtCytb, ΔGtIsp, and 24-HN-1 mutants may be related to this (Hawke, 1994; Henson et al., 1999). Laccase can catalyse the polymerization of 1,8-dihydroxynaphthalene into high-molecular-weight melanin (Litvintseva and Henson, 2002). The results of the laccase enzyme activity assay showed that the activity in resistant strain 24-HN-1 was higher than that of the mutants ΔGtCytc_1, ΔGtCytb, and ΔGtIsp.

Peroxidase is a major scavenger of ROS. ROS is a collective term that includes the superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and the hydroxyl radical (HO^-; Dautreaux and Toledano, 2007). ROS are generated by the incomplete reduction of molecular oxygen during the process of oxidative phosphorylation.
with carabrone at EC$_{30}$ in vivo (40%–50% and 70%–80%, respectively), except for ΔGtLsp and 24-HN-1 (Figure 6a), which may be insensitive to carabrone. Compared with the control, the activity of complex III in all strains was significantly decreased following treatment with carabrone at EC$_{20}$ and EC$_{50}$ in vitro (40%–50% and 70%–80%, respectively), except for ΔGtLsp and 24-HN-1 (Figure 6a), which may be insensitive to carabrone. In contrast, the activity of complex III in all strains was significantly increased following treatment with carabrone at EC$_{20}$ in vivo (ranging from 5% to 38%), except for 24-HN-1 and OEIsp, and complex III activity was decreased in all strains after exposure to carabrone at EC$_{50}$ in vivo (ranging from 11% to 64%; Figure 6b). The carabrone may induce mitochondrial alternative oxidase, which leads to increased activity of respiratory chain complex III at low concentrations in vivo. The results showed that the targeting of the iron–sulphur protein unit encoded by GtLsp was sensitive to carabrone; this observation provides a valuable clue to the possible target of carabrone activity and could form the basis for further research.

There is a considerable amount of evidence that shows a decline in oxygen consumption and alterations in electron transport chain complex activities with inhibition of the respiratory chain complex and increasing age (Schwarze et al., 1998; Ferguson et al., 2005). In this study, the oxygen consumption rates of mitochondrial respiration in all strains were higher with respect to the control when treated with carabrone at EC$_{20}$. The findings herein are consistent with the results that the mitochondrial alternative oxidase was activated when the respiratory chain was inhibited by carabrone at a low concentration. The results showed that the rates of mitochondrial respiration of all strains, except for ΔGtCytb, were significantly inhibited (ranging from 57% to 95%) when treated with antimycin A (20 μg/ml), which is consistent with earlier reports suggesting that antimycin A is an inhibitor of cytochrome b in mitochondrial respiratory chain complex III (Gao et al., 2002; Kotiaho et al., 2008). The rates of mitochondrial respiration of all the strains, except for ΔΔGtLsp, were significantly inhibited following treatment with carabrone at EC$_{80}$ (ranging from 57% to 81%; Figure S1).

In summary, carabrone constitutes a promising new environmentally friendly biofungicide, though the mode of action on G. tritici requires further clarification. We successfully located the site of action of carabrone to the mitochondria through immunogold labeling. Combined with the results of previous studies, we silenced and overexpressed the genes GtCytc$_1$, GtCytb, and GtLsp of the mitochondrial respiratory chain complex III in G. tritici. Based on the results, we conjecture that carabrone possibly influences the mitochondrial respiratory chain complex III in G. tritici by binding to the surface of the iron–sulphur protein; maybe it is similar to stigmatellin and the 2-hydroxyquinone analogues that occupy the region of the Q$_o$ site close to the docking interface of the iron–sulphur protein (Fisher and Meunier, 2005). Thus, these findings provide essential insights for clarifying the mechanism of action of carabrone in G. tritici. Further studies are required to elucidate the interaction between carabrone and iron–sulphur proteins.

4 | EXPERIMENTAL PROCEDURES

4.1 | Fungal strains and media

The G. tritici wild-type strain ACCC30310 was provided by the collection Agricultural Culture Collection of China (ACCC, Beijing, China, http://www.accc.org.cn/search/accc/show.asp?jzbc=30310). Strain ACCC30310 of G. tritici was used for transformation in this study.

The carabrone-resistant strain 24-HN-1 was obtained through preliminary breeding in the laboratory. The EC$_{50}$ of 24-HN-1 was 183.36 μg/ml, the EC$_{30}$ of the wild-type strain was 45.36 μg/ml, and the resistance level reached 4.06 times.

All strains (Table 1) were grown on PDA at 25 °C for 5 days. Liquid cultures were routinely grown in 100 ml PDB at 25 °C with shaking at 175 rpm for 5 days. All strains were stored at ~80 °C by cryopreservation in 30% glycerol solution. Sequence data of G. tritici from this article were deposited in GenBank with the following accession numbers: GtCytc$_1$ (XM_009219003.1), GtCytb (XM_009220561.1), GtLsp (XM_009221216.1), and GtLac (AJ437319.1), and the housekeeping gene 18S rRNA (U08320).

4.2 | Preparation of a monoclonal antibody

Immunogen (T-BSA) was synthesized according to the method described by Wang et al. (2017b). All mouse experimental procedures were performed in accordance with the Regulations for the Administration of Affairs Concerning Experimental Animals...
Female BALB/c mice (6 weeks old), purchased from the Xi'an Jiaotong University School of Medicine (Shaanxi, China), were immunized with immunogen (T-BSA). Immunization of the animals and titres of the antisera were monitored by indirect ELISA, as described by Wang et al. (2017b). The monoclonal antibody was prepared according to the method described by Wang et al. (2017a) with slight modifications. Spleen lymphocytes and myeloma cells Sp2/0 were prepared according to standard procedures in a sterile environment (Köhler and Milstein, 1975) and were fused at a 5:1 ratio using polyethylene glycol (PEG) as the fusing agent. After 9 days of culture, the positive hybridoma was screened by the ic-ELISA and then cloned three times by limiting the dilution and cryopreservation. In addition, the hybridomas were collected to produce ascites via intraperitoneal injection. The antibodies were purified by octanoic acid-saturated ammonium sulphate.

Appropriate dilutions were determined by testing a series of concentrations of antibodies and coating antigens by checkerboard titration. The sensitivity and specificity of the ELISAs were evaluated by IC_{50} and cross-reactivity. Several compounds that were structurally related to carabrone compounds, including carabrol and γ-phenyl-α-methylene-γ-butyrlactone, were selected for cross-reactivity testing. The concentrations of the standard solutions of the compounds covered a range from 1.25 to 4,000 ng/L. The cross-reactivity values were calculated as follows: cross-reactivity (%) = (IC_{50} of carabrone/IC_{50} of the other compound) × 100.

4.3 Immunogold subcellular localization

Mycelia (1 g fresh weight) from a 3-day-old culture of G. tritici were harvested in PDB and then transferred to 100 ml of PDB containing either carabrone (EC_{750}) or nothing (blank control) and incubated at 175 rpm at 25 °C for 6, 12, and 48 hr, respectively. A small amount of hyphae was added to a 1.5-ml centrifuge tube containing 1 ml of 0.2% agar. The agar (3 × 5 × 5 mm) was cut with a sterile scalpel once the hyphae were combined and the agar had solidified. The samples were fixed, dehydrated, and embedded according to the method described by Gao et al. (2005).

Immunogold labelling was carried out according to the method described by Kang and Buchenauer (2002, 2003) with slight modifications. Ultrathin sections of the samples were collected on nickel grids, inverted on droplets of ultrapure water, and wetted for 1 min. The ultrathin sections were incubated with blocking solution (5% goat serum; Solarbio) for 20 min, then incubated with the primary antibody diluted at 1:1,000 in the blocking solution for 1 hr at room temperature, and finally washed in phosphate-buffered saline (PBS). The sections were incubated with the secondary antibody (12 nm colloidal gold-affinipure goat anti-mouse IgG [H + L]; Bioleaf Biotech) and diluted 1:100 in PBS for 30 min, following which the sections were washed with PBS and rinsed in distilled water. After contrasting with uranyl acetate and lead citrate, the sections were examined with a 10 CR electron microscope (Zeiss) at 60 kV.

4.4 Construction of hairpin RNA silencing and overexpression vectors

To silence GtCytC1 expression in the wild-type strain, a 375 bp gene fragment was amplified with the primer pair ZGtCytC1-F/R, purified, and inserted into the Xhol/HindIII site of pSilent-1 (Figure S2a). The resulting plasmid was named pSilent-GtCytC1-up. The same fragment of GtCytC1 was amplified with the primer pair FGtCytC1-F/R, purified, and inserted into the KpnI/SphI site of pSilent-GtCytC1-up (Figure S2b). The recombinant silencing plasmid was designated as pSilent-GtCytC1, purified, and transferred into the wild-type strain using the PEG-mediated transformation method according to Wang et al. (2018). The hairpin RNA silencing plasmids of GtCytb (387 bp) and GtIsp (389 bp) were constructed with the same strategy and verified by PCR with the primer pair PScheck 1F/R (Figure S3). The mutants ΔGtCytC1, ΔGtCytb, and ΔGtIsp were verified by PCR with the primer pair PScheck 1F/R (Figure S4).

For the overexpression assays, a 978 bp fragment containing the entire GtCytC1 gene was amplified with the primer pair OECytC1-F/R and cloned into pFL2 (Zhou et al., 2011) using a yeast in vivo homologous recombination approach, as previously described (Bruno et al., 2004). The resulting overexpression vector was transformed into the wild-type strain ACCC30310. The overexpression vectors of GtCytb (1,485 bp) and GtIsp (726 bp) were constructed with the same strategy and verified by PCR with the primer pair pFLcheck-F/R (Figure S3). The primer pairs used to amplify the target regions are listed in Table S3. The mutants OECytC1, OECytb, and OEIsp were verified by PCR with the primer pair pFLcheck-F/R (Figure S4).

4.5 DNA and RNA isolation

The mycelia of G. tritici were cultured according to the method described by Rachdawong et al. (2002). The wild-type strain (ACCC30310) and the mutants 24-HN-1, ΔGtCytC1, ΔGtCytb, ΔGtIsp, OECytC1, OECytb, and OEIsp were grown on Petri dishes containing PDA at 25 °C for 7 days before harvesting in sterilized distilled water. The mycelia were then transferred into a conical flask containing 100 ml PDB and incubated on a rotary shaker (175 rpm) at 25 °C for 5 days. Genomic DNA of the eight strains was extracted using a Plant Genomic DNA Kit (CWBIOL). Total RNA of the strains was extracted using the RNAsimple Total RNA Kit (Tiangen) according to the manufacturer’s instructions. The concentrations of DNA and RNA were determined using an ND-1000 spectrophotometer (NanoDrop), and the isolated DNA and RNA were stored at −80 °C prior to analysis.
4.6 | RT-qPCR

The RNA was extracted as described above and reverse transcription was performed according to the method described by Wang et al. (2017c). RT-qPCR analysis was performed on an Applied Biosystems QuantStudio 6 Flex Real-Time PCR System (Thermo Fisher Scientific) using SYBR Premix Ex Taq II (Takara) prepared according to the manufacturer’s instructions. The gene for laccase (GtLac) and the mitochondrial respiratory chain complex III-related genes of G. tritici cytochrome c₁ (GtCytC₁), cytochrome b (GtCytb), and Rieske protein (GtIsp) were selected. The 18S rRNA gene of G. tritici was used as a reference. Specific oligonucleotide primers for the amplification of GtLac, GtCytC₁, GtCytb, GtIsp, and 18S rRNA from G. tritici are shown in Table S3. Samples were prepared for RT-qPCR in a total reaction volume of 20 μl containing 10 μl of SYBR Premix Ex Taq II, 0.8 μl of PCR forward primer (10 μM), 0.8 μl of PCR reverse primer (10 μM), 0.4 μl of ROX reference dye II, 2 μl of cDNA sample or DNA sample, and 6 μl of RNase-free dH₂O. The thermal profile for the RT-qPCR was 30 s at 95 °C followed by 40 cycles of 5 s at 95 °C and 34 s at 60 °C. The expression change (ΔCt) of a target gene (Tg) based on the cycle threshold (Ct) was calculated as fold change = 2^(-ΔCt_Tg − ΔCt_control). Each reaction was run in triplicate with at least three independent replicates.

4.7 | Assays for mycelial growth and stress responses

The wild-type strain and the 24-HN-1, ∆GtCytb, ∆GtIsp, OECytb, and O EIsp mutants were routinely cultured on PDA at 25 °C. Each plate was inoculated with a 5-mm diameter mycelial plug obtained from the edge of a 7-day-old colony. The mean colony diameters (minus the diameter of the inoculation plug) were measured at 1, 2, 3, 4, 5, and 6 days. For testing sensitivities to carabrone and oxidative stress, mycelial growth was assayed after incubation at 25 °C for 7 days on PDA plates with 100 μg/ml carabrone and 10 mM H₂O₂. There were three replicate plates for each treatment and the experiment was performed three times. Hyphal tip growth and branching of the wild-type progenitor, 24-HN-1, ∆GtCytC₁, GtCytb, GtIsp, OECytC₁, OECytb, and O EIsp were examined by scanning electron microscopy (Hitachi). The eight strains were grown on a thin layer of water agar with or without 100 μg/ml carabrone or 10 mM H₂O₂ for 3 days, according to the method described by Cleary et al. (2013).

4.8 | Determination of intracellular melanin

Melanin concentration was estimated by the method of Frederick et al. (1999). For preparation of melanin for the standard curve, eight agar plugs (5 mm diameter) of the wild-type strain (ACCC30310), selected from the leading edge of a colony on PDA, were inoculated into a conical flask containing 100 ml of PDB and incubated on a rotary shaker (175 rpm) at 25 °C for 5 days. The cultures were filtered through sterile cheesecloth and acidified with 4 M HCl. After allowing precipitation at 4 °C for 4 days, melanin was recovered by centrifugation at 5,000 × g for 15 min. The pellet was washed three times with 0.1 M HCl followed by three washes with deionized water, and then lyophilized overnight and stored at −20 °C. A standard melanin curve was prepared by weighing duplicate 50, 100, 150, 200, 250, and 300 μg samples into 2-ml tubes. One millilitre of Azure A (Sigma) solution (33 mg/ml in 0.2 M HCl) was added to each tube, allowed to stand for 30 min, and then filtered through a 0.45-μm syringe filter. The melanin concentration was determined by a decrease in the filtrate absorbance of Azure A at 628 nm. Melanization was determined at 3, 4, 5, 6, and 7 days from 100 ml of PDB in mutants 24-HN-1, ∆GtCytb, ∆GtIsp, OECytb, OECytb, O EIsp, and the wild-type. Mycelia were collected by filtration through sterile cheesecloth and lyophilized overnight. Triplicate 2 mg samples of the dried mycelia were assayed for melanin content, as described for the standard melanin curve.

4.9 | Measurement of enzyme activity

The activity of peroxidase, laccase, and mitochondrial respiratory chain complex III was assessed spectrophotometrically using a SpectraMax plus 384 (Molecular Devices). Peroxidase and laccase activity were measured using the method of Chi et al. (2009). Enzyme activity was estimated using culture filtrate from 5-day-old PDB of the wild-type progenitor, ∆GtCytb, ∆GtCytb, ∆GtIsp, 24-HN-1, OECytb, OECytb, and O EIsp. Mycelia were completely removed by filtration and centrifuged at 5,000 × g for 1 min at 4 °C. For measurement of peroxidase and laccase activity, a reaction mixture (1 ml) containing 50 mM acetate buffer (pH 5) and 10 mM 2,2′-azinobis-(3-ethylbenzthiazoline-6-sulphonate) (ABTS; Sigma) was mixed with the culture filtrate (200 μl) and incubated at 25 °C for 5 min with or without 3 mM H₂O₂. Absorbance was evaluated at 420 nm. The activity of the respiratory chain complexes III was determined spectrophotometrically at 550 nm according to Wang et al. (2017c).

4.10 | Plant infection assays

Take-all responses of the wild-type progenitor, 24-HN-1, ∆GtCytC₁, ∆GtCytb, ∆GtIsp, OECytC₁, OECytb, and O EIsp were evaluated by inoculation with each isolate. Wheat seeds were surface sterilized in 5% sodium hypochlorite solution for 5 min, followed by two rinses in deionized water. Each 2-day germinated seed was placed on top of one agar plug with each isolate culture and then covered with 2 cm of thick sand. Agar plugs (5 mm diameter) were taken from the growing edges of the 7-day-old PDA cultures of each isolate (controls used sterile PDA plugs). There were three replicates of each isolate and each replicate consisted of nine wheat seedlings. At 21-25 days after inoculation, plants
were lyophilized overnight and roots and shoots were weighed and measured (Frederick et al., 1999). For infected seedlings, the severity of disease was examined by measuring the lengths of all lesions. The percentage of the root area affected (with lesion) was also calculated using the formula % root area affected = 100 × (lesion length/total root length) (Chng et al., 2005).

4.11 | Rates of mitochondrial respiration

Rates of mitochondrial respiration were determined polarographically using a Clark-type oxygen electrode connected to a computer-operated Oxygraph control unit (Hansatech Instruments) according to the method described by Ferguson et al. (2005). Mycelia of the wild-type progenitor, 24-HN-1, ∆GtCytb, ∆GtIsp, OECytb, and OELsp were grown on Petri dishes containing PDA at 25 °C for 7 days before harvesting in sterilized distilled water. The mycelia were then transferred into a conical flask containing 100 ml of PDB and incubated on a rotary shaker (175 rpm) at 25 °C for 5 days. Carabrone (control, EC<sub>20</sub> EC<sub>50</sub>) was then added to the flask with the wild-type progenitor, ∆GtCytb, ∆GtIsp, OECytb, 24-HN-1, OECytb, OECytb, and OELsp, and the mycelia were collected at 12 hr. Mitochondria were prepared according to Wang et al. (2017c). For all the experiments, the temperature was maintained at 28 °C and the total reaction volume was 2 ml. Freshly isolated mitochondria (800 µg protein) were added to the reaction medium (0.4 M mannitol, 0.2 M sucrose, 10 mM KCl, 10 mM MgCl<sub>2</sub>, 50 mM potassium phosphate, 10 mM Tris, pH 7.4), which was then measured for 8 min.

4.12 | Data analysis

Statistical analysis was performed using SPSS v. 19.0. Differences between groups were evaluated by one-way analysis of variance (ANOVA). p < .05 was considered statistical significance.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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