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ORIGINAL ARTICLE

Silencing *GhCOI1* in *Gladiolus hybridus* increases susceptibility to *Alternaria brassicicola* and impairs inducible defenses

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Abstract

Alternaria brassicicola is a necrotrophic pathogenic fungus that severely affects both the yield and quality of *Gladiolus* cut flowers and cormels. The jasmonate signaling pathway plays an important role in plant disease resistance. The protein CORONATINE INSENSITIVE 1 (COI1) is a key regulator of this pathway, and the *COI1* gene can modulate jasmonate signaling-induced defenses against necrotrophic fungi. Here, we characterize a *COI1* gene from *Gladiolus*, *GhCOI1*. *GhCOI1* transcript levels were greatest in the leaves, followed by the roots. A low MeJA concentration promoted *GhCOI1* expression, which remained high after 6 h. Silencing *GhCOI1* via VIGS reduced *GhCOI1* transcript levels. The length of fungal hyphae and amount of fungal DNA were significantly greater in silenced plants than in control plants. Moreover, GhCOI1 was necessary for the induction of SOD, POD, PPO, and CAT in the *Gladiolus* defense response to *A. brassicicola* infestation. *GhCOI1* silencing reduced the accumulation of H_2O_2, allowing the fungus to enter leaf tissue through stomata and cause necrosis directly. Taken together, these results suggest that GhCOI1 is an essential signaling component that controls the JA-regulated defense response against *A. brassicicola* in *Gladiolus* plants.

Key message

The results of this study reveal that silencing *GhCOI1* reduced the disease resistance of *Gladiolus*

Keywords *Alternaria brassicicola* · CORONATINE INSENSITIVE 1 · *Gladiolus* · Virus-induced gene silencing (VIGS)

Introduction

Gladiolus is vulnerable to many diseases that severely affect both the yield and quality of its cut flowers and corms, causing extensive economic losses (Gao and Wu 2008; Li et al. 2007; Liu 2008; Pieterse et al. 2009; Pratibha et al. 2014). Pathogens that cause disease in *Gladiolus* include fungi, viruses, bacteria, and red spiders (Jiao and Guo 1999). The fungi that damage *Gladiolus* are mainly necrotrophic pathogenic fungi, among which *Alternaria brassicicola* is a typical pathogen that infects the leaves of plants, leading to leaf spot disease (Si and Si 2007). *A. brassicicola* can infect hosts directly or can enter through wounds or stomata (Sharma et al. 2014).

Numerous studies have shown that jasmonic acid (JA) is involved in plant growth and development (Cheng et al. 2009; Schommer et al. 2008), with important regulatory roles in the response to biotic and abiotic stresses (Browse and Howe 2008; Reymond and Farmer 1998; Farmer et al. 1992; Lorenzo et al. 2003; Stintzi et al. 2001). In addition, JA signaling is involved in response to diseases caused by necrotrophic pathogens. The JA signaling pathway begins with the recognition of hormone signals by the protein CORONATINE INSENSITIVE 1 (COI1), a JA receptor and key regulator of the JA signal transduction pathway

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(Katsir et al. 2008). The JA pathway is blocked in the *Arabidopsis* mutant *coi1*, resulting in enhanced susceptibility to *A. brassicicola* (Thomma et al. 1998). Furthermore, silencing of the *COI1* gene in rice plants by virus-induced gene silencing (VIGS) has been shown to reduce the activity of peroxidase (POD) and polyphenol oxidase (PPO) by 48.5% and 27.2%, respectively; the resistance of rice plants to rice leafroller also significantly decreased when *COI1* was silenced, suggesting that the *COI1* gene is vital for regulating JA-mediated resistance (Ye et al. 2012).

Differently, the resistance of *Gladiolus* to *A. brassicicola* has not been reported, and the overall role of the *COI* gene in disease resistance also remains unclear. In this study, we cloned a *COI* gene from *Gladiolus*, *GhCOI1*, and measured its expression in different organs. We also analyzed the changes in *GhCOI1* expression after methyl jasmonate (MeJA) treatment, verified the role of *GhCOI1* in the JA signaling pathway and explored the ability of *A. brassicicola* to infect *Gladiolus*. We further silenced the *GhCOI1* gene with VIGS and analyzed the *Gladiolus* defense response. Finally, we clarified the effect and function of *GhCOI1* in the susceptibility of *Gladiolus* to leaf spot disease. Our results provide a theoretical basis for improving the quality of both cut flowers and cormels.

Materials and methods

Plant materials and growth conditions

Gladiolus hybridus cv. 'Rose Supreme' was planted in the Science Research Garden of China Agricultural University. Samples were collected at 26 weeks after planting to determine the expression profile of the *GhCOI1* gene. Cormels (Fig. S1) with diameters of 1.0 cm were used as materials for VIGS assays. The cormels were cultivated in a greenhouse (22 °C temperature, 16 h/8 h light/dark photoperiod, 54 μmol m^{-2} s^{-1} illumination intensity). Four-week-old plantlets grown in the greenhouse (one plant per pot) were individually sprayed with 1 mL of MeJA that consisted of 0.01% Tween 20.

Isolation and sequence analysis of *GhCOI1*

Total RNA was extracted from *Gladiolus* cormels via an RNA extraction reagent kit (Tiangen, Beijing, China), and cDNA was synthesized using Moloney murine leukemia virus (M-MLV) reverse transcriptase (TaKaRa, Shiga, Japan) and the oligonucleotide adapter primer AP1 (Table 1). Rapid amplification of cDNA ends (RACE) was used for *GhCOI1* gene cloning, and nested PCR was performed according to the manufacturer's protocol (Clontech, CA). The protein characteristics were assessed via ExPASy

Table 1 Primers used for *GhCOI1* cloning and in gene expression analysis by qRT-PCR

Gene name	Primer name	Sequence (5′–3′)
GhCOI1	Primers for gene cloning	
	AP1	CCAGTGAGCAGAGTGACGAGGACTCGAGCTCAAGC$(T)_{17}$
	AP2	CCAGTGAGCAGAGTGACG
	AP3	GAGGACTCGAGCTCAAGC
	DP-*COI1*-F1	GGVAARCCHMGMGCNGCBATGT
	DP-*COI1*-F2	ANGHKYTBAAGCTNGAYA AGTG
	DP-*COI1*-R1	ATGCYCTRTADCCYTGYACCCA
	SP-*COI1*-F3	CGGAGTTGCCCCTGGATAATGG
	SP-*COI1*-F4	TGTCGGAAACTGCAGAAACTGGA
	SP-*COI1*-R2	CCTTCTCAGTAATGGTGCTTTCTTCC
	SP-*COI1*-R3	GCAAGAACGAGCAATGAGGGTGAGGG
	SP-*COI1*-F5	ATGGAGAACGGCAGTAGTAGTA
	SP-*COI1*-R4	GGACTAGTCACCATCTGGGCAATCTGTCCTTTG
	Primers for vector construction for VIGS in gladiolus	
	*Xba*I_ for.	GCTCTAGAGCTGGACAAAGGACAGATTGC
	*Sma*I_ rev.	TCCCCCGGGGTGTCCCAGAAAAAGCATATC
GhCOI1	Primers for qRT-PCR	
	qF1	GGTGGTATATCGGATGCGGCT
	qR1	CGAGGAATAGCAGAGGGCCA
GhActin	qF2	ACTGCAGAGCGGGAAATTGT
	qR2	CCAATCAGGGATGGCTGGAA

proteomics tools (http://au.expasy.org/tools/). Amino acid multiple alignments were performed by ClustalX 1.8 and BioEdit 7.0, and phylogenetic trees were constructed via MEGA 7.0 software.

Expression analysis of *GhCOI1* by qRT-PCR

Via an RNA extraction kit (Tiangen, Beijing, China), total RNA was isolated from samples in liquid nitrogen. The RNA samples were reverse-transcribed using a reverse transcriptase kit (BioTeke, Beijing, China). Four hundred nanograms of cDNA was used as a template for qRT-PCR via a KAPA™ SYBR® FAST qPCR kit (Kapa Biosystems, Woburn, MA, USA) and an Applied Biosystems StepOnePlus™ real-time PCR system. The *Gladiolus* actin gene (GenBank Accession No. JF831193) was used as a control in *Gladiolus*. The PCR procedure was performed according to the manufacturer's instructions. Each qRT-PCR assay was run in three biological replicates. The specific primers used for qRT-PCR are shown in Table 1, and the qRT-PCR procedure was performed as described previously (Seng et al. 2016). The relative transcription levels were calculated according to the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001).

Inoculation of *A. brassicicola* onto *Gladiolus* leaves

The spore/mycelium mixture (obtained from the Jianqiang Li Laboratory) was shaken at 100 rpm for 30–45 min at 26 °C, which facilitated the complete dispersion of the spores. Sterile gauze was folded into 5–6 layers, and the mixture was then filtered into a new triangular bottle. The new mixture was subsequently centrifuged at 10,000×*g* for 5 min at room temperature. *A. brassicicola* spores were collected in sterile water by suspension. The *A. brassicicola* spores were then diluted to a concentration of 1×10^6 cells/mL in sterile water, after which 0.2% Tween 20 was added as an emulsifier for adhesion to the leaf surface. The solution was sprayed evenly onto the upper and lower surfaces of the leaves of *Gladiolus* plants. The plants were completely covered with transparent plastic cloth and placed in an incubator (22 °C, 16 h/8 h light/dark photoperiod, 54 μmol m^{-2} s^{-1} illumination intensity).

Trypan blue staining

Gladiolus leaves inoculated with fungi were placed in 0.25 mg/mL trypan blue staining solution (lactic acid:glycerol:phenol:sterile water = 1:1:1:1) and infiltrated under vacuum for 20 min. The leaves were then heated for 1–3 min in decolorizing solution (95% ethanol:lactic phenol = 2:1). After the mixture cooled, the leaves were rinsed in 50% ethanol solution for thorough decolorization. The samples were subsequently observed under a microscope, and ImageJ software was used to measure the lengths of hyphae and the necrotic areas of cells.

3,3-Diaminobenzidine (DAB) staining

Gladiolus leaves inoculated with fungi were placed in 1 mg/mL DAB staining solution (pH 5.5) for 20 min, after which the leaves were heated in 95% ethanol solution for 2 min. After the mixture cooled, the leaves were rinsed in 50% ethanol solution for thorough decolorization (Liao et al. 2019). The samples were then observed under a microscope, and ImageJ software was used to measure the stained area.

Superoxide dismutase (SOD), catalase (CAT), POD and PPO activity assays

A 0.5 g leaf sample from tobacco rattle virus (TRV) control and silenced plants was harvested and extracted in liquid nitrogen, followed by homogenization with 10 mL of extraction buffer consisting of 100 mM potassium phosphate buffer (pH 7.0) containing 1 mM EDTA. The mixture was filtered through 4 layers of cheesecloth and centrifuged at 12,000×*g* for 20 min at 2–4 °C. The supernatant was then used as the enzyme extract. SOD activity was assayed by the photochemical nitro blue tetrazolium (NBT) method, in which 3 mL of reaction mixture contained 100 mM potassium phosphate buffer (pH 7.0), 13 mM methionine, 0.1 mM EDTA, 50 mM sodium carbonate and 25 mM NBT and 0.1 mL of enzyme. SOD activity was determined according to the method of Beyer and Fridovich (1987): one unit of SOD activity was defined as the amount of enzyme that inhibited the photoreduction of NBT by 50% at 560 nm of optical density measured with a spectrophotometer (Hitachi U-2000). CAT activity was assayed by tracking the consumption of hydrogen peroxide (H_2O_2) at 240 nm for 3 min (Aeby 1984). The 3 mL reaction mixture consisted of 100 mM phosphate buffer (pH 7.0), 5 mM H_2O_2, and 50 μL of enzyme extract, and the final volume was brought to 3 mL with water. One unit of CAT consumes 1 nmol of H_2O_2/min under the assay conditions. POD activity was measured by following the change in absorbance at 470 nm due to guaiacol oxidation (Polle et al. 1994). The activity was assayed for 1 min in a 3 mL reaction solution comprising 100 mM potassium phosphate buffer (pH 7.0), 20 mM guaiacol, 10 mM H_2O_2 and 50 μL of enzyme extract. PPO activity was assayed in a 4 mL reaction solution composed of 100 mM potassium phosphate buffer (pH 7.0), 0.2 M catechol and 50 μL of enzyme extract at 420 nm.

Silencing of the *GhCOI1* gene in *Gladiolus*

Silencing of *GhCOI1* in *Gladiolus* by VIGS was performed as described previously (Zhong et al. 2014). A specific fragment of *GhCOI1* was inserted into a pTRV2 vector to construct a pTRV2-*GhCOI1* plasmid. The resulting pTRV1, pTRV2 and pTRV2-*GhCOI1* plasmids were transformed separately into *Agrobacterium tumefaciens* GV3101, which was cultured at 28 °C overnight in Luria–Bertani medium supplemented with 50 μg/mL kanamycin and 50 μg/mL rifampicin. The cultures were collected by centrifugation at 5000 rpm for 15 min, after which the cells were resuspended in infiltration buffer (10 mM $MgCl_2$, 10 mM 2-[N-morpholino]ethanesulfonic acid [MES], and 200 mM acetosyringone) at a final optical density at 600 nm (OD600) of approximately 2.0. Holes were poked into the top and bottom of cormels whose diameter was 1.0 cm. After infection, the cormels were grown in pots in a greenhouse (22 °C, 16 h/8 h light/dark photoperiod).

Results

Isolation and sequence analysis of the *GhCOI1* gene

The full-length *GhCOI1* gene was 2603 bp and contained an open reading frame (ORF) that was 1842 bp in length; this gene encoded a polypeptide of 613 amino acids with a predicted molecular mass of 69.46 kD, and the isoelectric point is predicted to be 5.89. The molecular formula of the GhCOI1 protein is $C_{3054}H_{4875}N_{855}O_{913}S_{40}$. This protein is soluble, with a stability coefficient of 53.83. There is no transmembrane domain, and the protein does not belong to a family of transmembrane proteins.

Analysis of the functional domain structure of GhCOI1 showed that this protein is a member of a class of F-box proteins (Fig. 1). GhCOI1 contains two important types of characteristic features: one F-box domain located in the N-terminal region and 9 leucine-rich repeat (LRR) motifs. The protein contains 6 JA-binding sites (Arg^{102}, Arg^{367}, Glu^{369}, Val^{406}, Arg^{428}, and Arg^{515}), which is consistent with AtCOI1 of *Arabidopsis*. In addition, GhCOI1 has 6 phosphorylation sites, including 4 serine phosphorylation sites (Ser^{14}, Ser^{16}, Ser^{21}, and Ser^{25}), 1 threonine phosphorylation site (Thr^{359}) and 1 tyrosine phosphorylation site (Tyr^{476}).

Phylogenetic analysis revealed that GhCOI1 is closely related to SiCOI1 from *Sesamum indicum* (Fig. 2). Phyre2 (http://www.sbg.bio.ic.ac.uk/phyre2/htmL) was used to analyze the tertiary structure of the COI proteins. The results showed that the tertiary structures of GhCOI1 and AtCOI1 are highly similar (66%). In addition, the *Arabidopsis* auxin receptor AtTIR1 is closely related to AtCOI1, and phylogenetic analysis grouped these proteins onto the same branch. AtTIR1 also contains LRR and F-box domains. The tertiary structures of GhCOI1 and AtTIR1 are 33% similar (Fig. 3).

Expression profiles of *GhCOI1*

The relative expression levels of *GhCOI1* were determined by qRT-PCR assays. *GhCOI1* was expressed constitutively in different organs at distinct levels. The highest levels of *GhCOI1* mRNA were detected in the leaves, followed by the roots; relatively low levels were detected in the flowers, and the lowest levels were detected in the cormels (Fig. 4a).

GhCOI1 expression in the leaves was evaluated at 12 h after the plants were sprayed with different concentrations of MeJA. The highest *GhCOI1* expression level, which was approximately 1.5-fold greater than the control level, was detected after treatment with 0.1 mM MeJA. However, compared with those in response to the control treatment, the changes in *GhCOI1* expression in response to 0.05 mM and 0.5 mM MeJA treatment were not significantly different (Fig. 4b). An appropriate concentration (0.1 mM) of MeJA promoted GhCOI1 expression, while high concentrations of MeJA (0.75 and 1.00 mM) repressed GhCOI1 expression.

GhCOI1 expression was further analyzed in the leaves of plants treated with 0.1 mM MeJA after 0, 3, 6, 9, 12, 24, 48 and 72 h. As shown in Fig. 4c, MeJA gradually induced the expression of *GhCOI1* during the first 6 h, after which time negative feedback on the expression of GhCOI1 was detected, with a relatively high level of transcript maintained. Similar results have been detected in rice and grapes treated with MeJA (Ye et al. 2012; Wang et al. 2012), indicating that *GhCOI1*, *OsCOI1* and *VvCOI1* may have similar roles in the JA signaling pathway.

Silencing the *GhCOI1* gene reduced its expression in *Gladiolus*

Leaves of *Gladiolus* inoculated with *A. brassicicola* were observed via stereomicroscopy. We found that the pathogen successfully infected leaves and caused leaf spot disease. On the 8th day after inoculation, yellow-green and irregularly shaped lesions began to appear on the leaves. Twelve days later, the central color of the lesions had deepened to brown, and there were light green to yellow-green halos surrounding these lesions. By the 20th day, the yellow-green halos had gradually expanded over the surface of the leaves, and the dark-brown lesions had further deepened to a black-brown color. Additionally, adjacent lesions had fused into irregular round lesions (Fig. 6a).

To determine whether *GhCOI1* plays a role in disease resistance, we used VIGS to reduce its expression and observed the phenotypes of control and *GhCOI1*-silenced plants inoculated with *A. brassicicola* (Fig. 5a). The results showed that, when the control plants were treated with *A.*

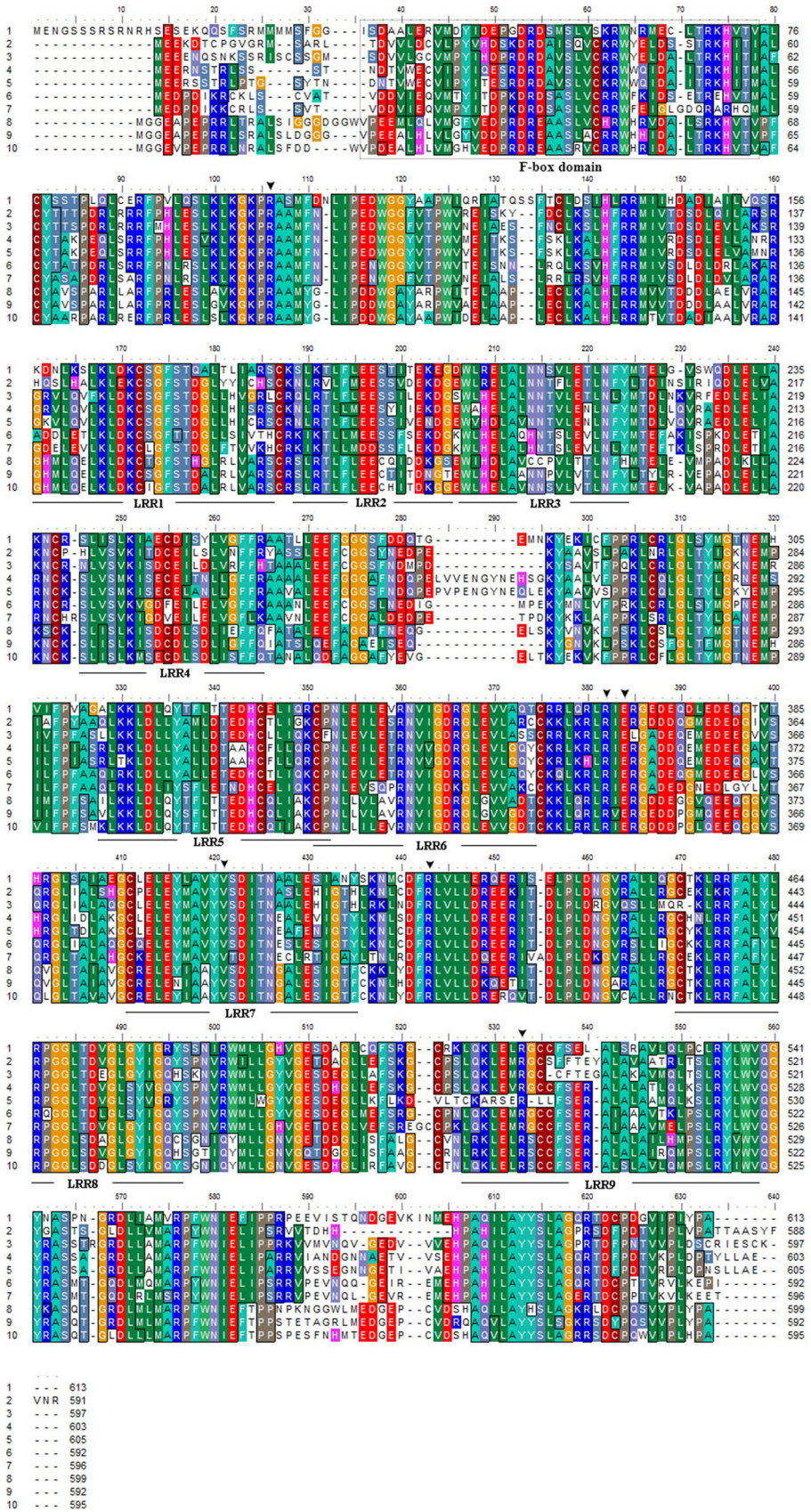

Fig. 1 Comparison of the predicted amino acid sequences of GhCOI1 and other plant COIs. Multiple sequence alignments of COI1 were performed using ClustalX 1.8 and BioEdit 7.0 software. Sequences for the following species were obtained from the NCBI database: 1, *Gladiolus hybrid*; 2, *Pisum sativum* (EF486643.1); 3, *Hevea brasiliensis* (EU136026.1); 4, *Lycopersicon esculentum* (AY423550.1); 5, *Nicotiana attenuata* (EF025087.1); 6, *Arabidopsis thaliana* (NM_129552.3); 7, *Brassica rapa* subsp. *chinensis* (GU263836.1); 8, *Zea mays* (NM_001156957.1); 9, *Triticum aestivum* (HM447645.1); and 10, *Oryza sativa* (AY168645.1). Black box: F-box domain; black lines: leucine-rich repeats (LRRs); black triangles: jasmonate-binding sites

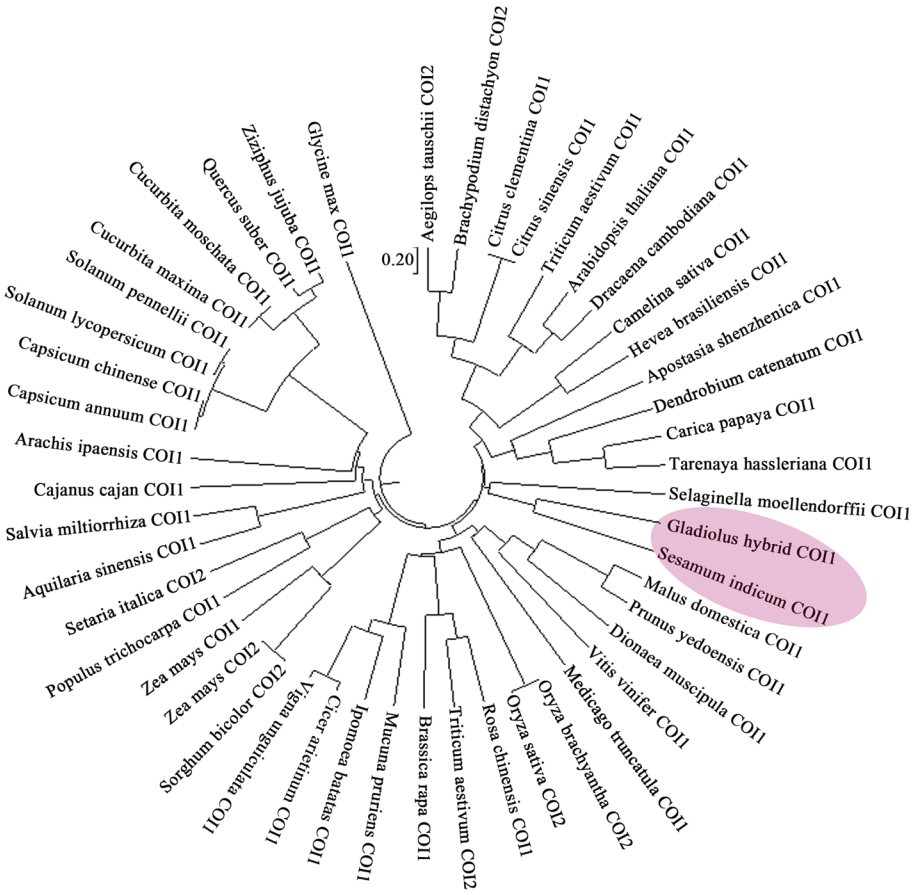

Fig. 2 Phylogenetic tree of COI proteins constructed on the basis of acid sequence similarity. The bootstrap values indicate the divergence of each branch, and the scale indicates the branch length. The COI protein sequences used for the construction of this tree were retrieved from the GenBank database

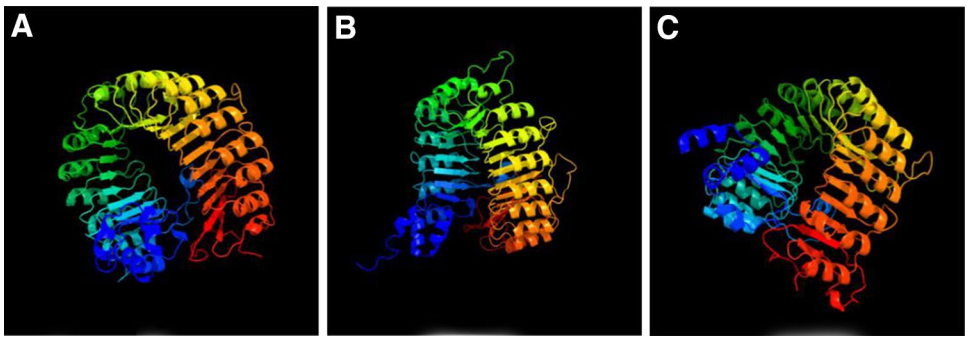

Fig. 3 Tertiary structural model of GhCOI1 (**a**), AtCOI1 (**b**) and AtTIR1 (**c**)

brassicicola, *GhCOI1* increased sharply in leaves during the first 6 h (0–6 h) but then decreased during the following 6 h (6–12 h). However, the expression of *GhCOI1* moderately increased again in the finally 60 h (12–72 h) (Fig. 5b). In the *GhCOI1*-silenced plants, *GhCOI1* barely responded to *A. brassicicola* (Fig. 5b). Moreover, *GhCOI1* expression levels in the leaves of silenced plants were 4.3-fold lower than those in the leaves of the control plants at 6 h. The expression pattern of *GhCOI1* in the leaves of control plants (Fig. 5b) was similar to that in the leaves of plants treated with 0.1 mM MeJA (Fig. 4c), suggesting that MeJA and *A. brassicicola* have similar roles in regulating *GhCOI1* expression.

Silencing the *GhCOI1* gene reduced plant disease resistance

Mycelial length is an important index of fungal growth. To determine whether fungal growth is affected by GhCOI1, the mycelial lengths of *A. brassicicola* inoculated onto *Gladiolus* leaves were analyzed (Fig. 6). The results showed that the length of mycelia on the leaves of both silenced and TRV

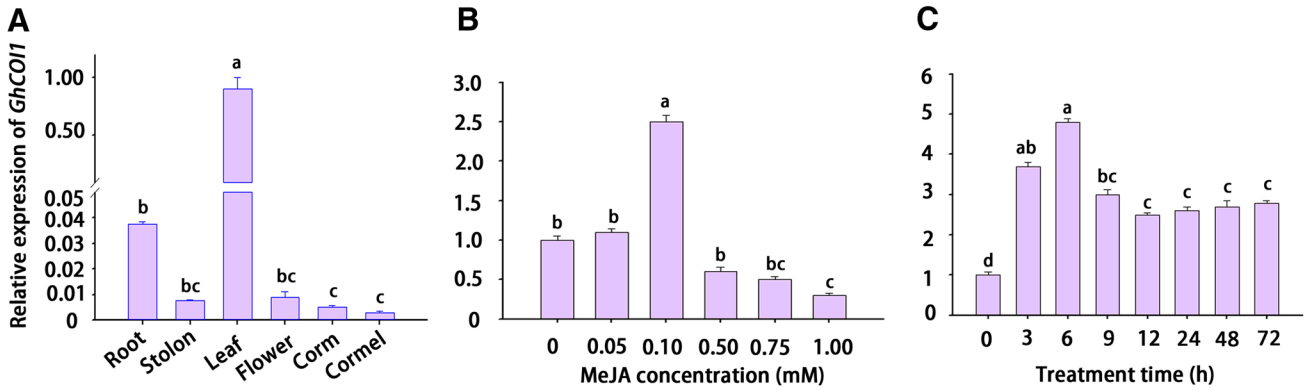

Fig. 4 *GhCOI1* transcript levels in *Gladiolus*. **a** Relative *GhCOI1* expression levels in various organs of *Gladiolus*. **b** Expression of *GhCOI1* in the leaves of plants treated with different concentrations of MeJA for 12 h. **c** Expression of *GhCOI1* in the leaves of plants treated with 0.1 mM MeJA after different incubation times. The error bars represent the SEs of three biological replicates (n = 3). The different letters indicate significant differences ($p < 0.05$; Duncan's multiple range test); the same letters indicate no significant differences

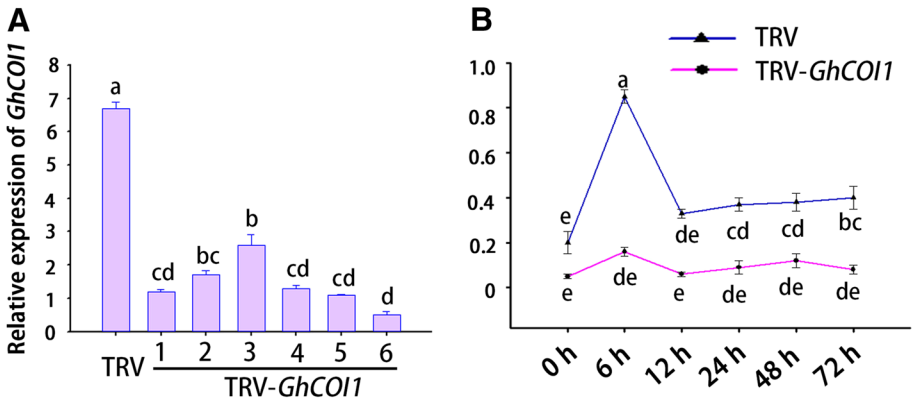

Fig. 5 Expression of *GhCOI1* in six *GhCOI1*-silenced plants inoculated with *Alternaria brassicicola*. **a** Expression of *GhCOI1* in the leaves of six *GhCOI1*-silenced independent lines. The error bar represents the SEs of three technical replicates. **b** *GhCOI1* is not induced in *GhCOI1*-silenced plants inoculated with *Alternaria brassicicola*. The error bar represents the SEs of three biological replicates of three independent plants. The different letters indicate significant differences ($p < 0.05$; Duncan's multiple range test); the same letters indicate no significant differences

control plants was less than 100 µm at 6 h after inoculation; however, the length of mycelia on the leaves of the silenced plants was slightly longer than those on the leaves of the TRV control plants. At 12, 24 and 36 h after inoculation, the lengths of the mycelia on the leaves of the silenced plants were 1.7-, 1.8- and 1.5-fold greater than those of the TRV control plants, respectively, suggesting that *GhCOI1* silencing promoted the growth of *A. brassicicola* on *Gladiolus* leaves.

To understand the effect of *GhCOI1* on disease, the amount of *A. brassicicola* DNA within the *Gladiolus* leaves was quantified by qRT-PCR. At 24 h after inoculation, the amount of fungal DNA within the silenced plants was 2.6-fold greater than that within the TRV control plants, and the difference was significant. After 36 h, the growth rates of the fungi within the control and silenced plants were 58.8% and 33.3%, respectively. Furthermore, at that time, the amount of fungal DNA within the silenced plants was still significantly (2.2-fold) greater than that within control plants (Fig. 7a). Overall, silencing *GhCOI1* increased fungal growth efficiency.

To further determine whether *GhCOI1* participates in *Gladiolus* resistance to *A. brassicicola*, the death of leaf cells infected by pathogens was analyzed by trypan blue staining. As shown in Fig. 7c, a small number of dead cells were observed in the leaves of both silenced and TRV control plants at 24 h after inoculation, and there was no significant difference in the sizes of the necrotic areas. After 36 h, the number of dead cells increased significantly, and the necrotic area was 1.8-fold greater in the silenced plants than in the TRV control plants, which was significantly different (Fig. 7b). These results show that the leaf necrotic

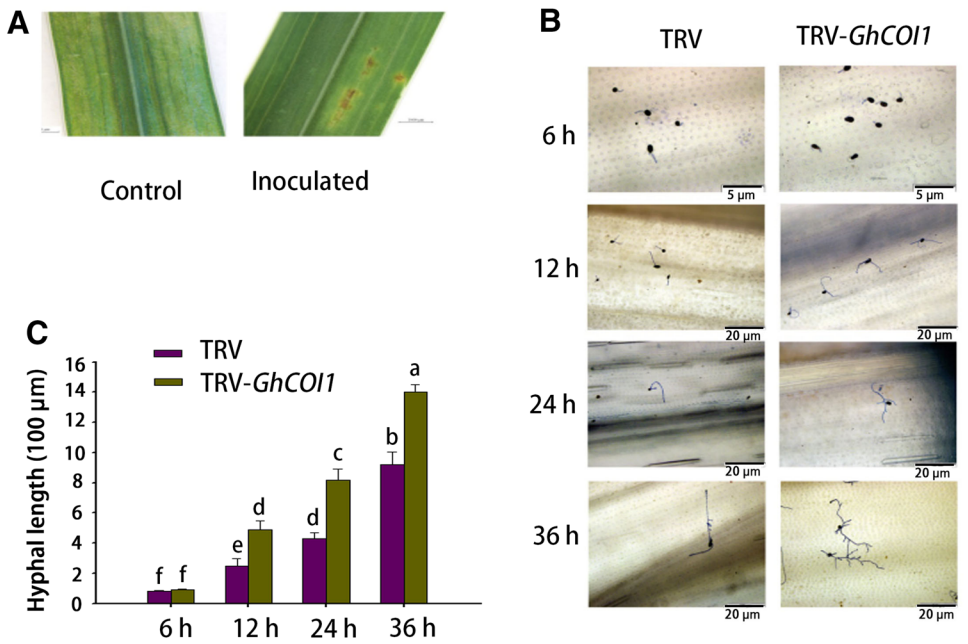

Fig. 6 Analysis of the length of hyphae on leaves after inoculation. **a** Leaf appearance at 20 days after inoculation. **b** Electron microscopy images showing hyphal length after leaf inoculation. The scale bars indicate 5 μm or 20 μm, as indicated. **c** Statistical analysis of hyphal length after leaf inoculation. The values represent the means, and the error bars represent the SEs of three biological replicates. The different letters indicate significant differences ($p < 0.05$; Duncan's multiple range test); the same letters indicate no significant differences

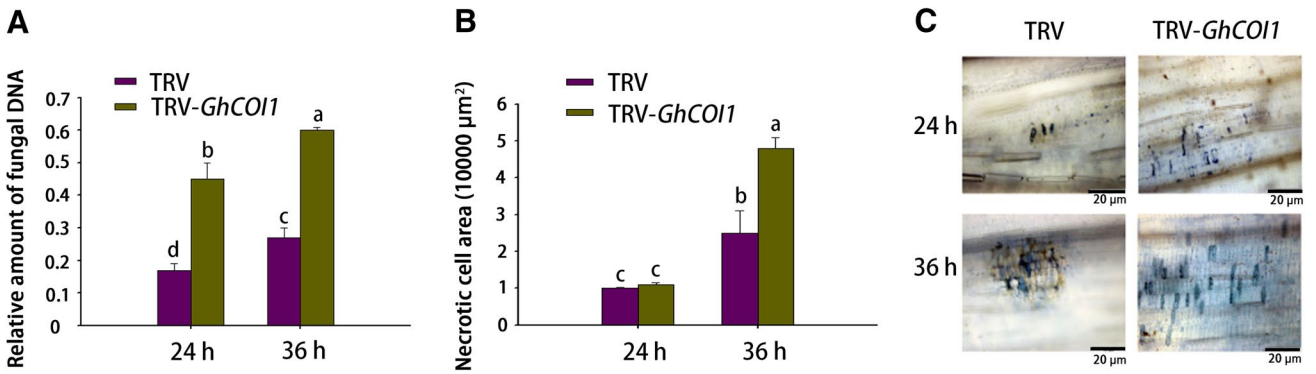

Fig. 7 Statistical analysis of fungal biomass and the leaf necrotic cell area. **a** Fungal biomass at 24 h and 36 h after leaf inoculation. DNA was extracted from the leaves of silenced and control plants (n = 3). **b** Statistical analysis of the leaf necrotic cell area of three different silenced plants. **c** Necrotic cells showing trypan blue staining of inoculated leaves. The values represent the means, and the error bars represent the SEs of three biological replicates. The different letters indicate significant differences ($p < 0.05$; Duncan's multiple range test); the same letters indicate no significant differences. (Color figure online)

area in the silenced and TRV control plants increased gradually with time after inoculation but that leaf necrosis was significantly greater in the silenced plants than in the TRV control plants. Therefore, *A. brassicicola* was better able to infect *GhCOI1*-silenced plants, and *GhCOI1* gene silencing rendered plants more susceptible to disease.

Silencing the *GhCOI1* gene reduced the activities of SOD, CAT, POD and PPO

The activities of the SOD, CAT, POD and PPO enzymes in TRV-*GhCOI1* plants were significantly lower than those in the TRV control plants (35.1%, 25%, 50.5% and 60% lower, respectively) (Fig. 8). These findings suggest that the *GhCOI1* gene is necessary for the induction of SOD, POD, PPO, and CAT enzyme activity in *Gladiolus* inoculated with *A. brassicicola* and that this gene may thus play a positive regulatory role in regulating enzyme activity.

Silencing the *GhCOI1* gene reduced H_2O_2 accumulation in plants

To investigate the relationship between *A. brassicicola* infection and H_2O_2 accumulation in leaves, the accumulation of H_2O_2 in the leaves was assessed by DAB staining, and the results were analyzed with ImageJ software. As illustrated in Fig. 9a, H_2O_2 accumulated in the leaves of both silenced plants and TRV control plants at 24 h after inoculation.

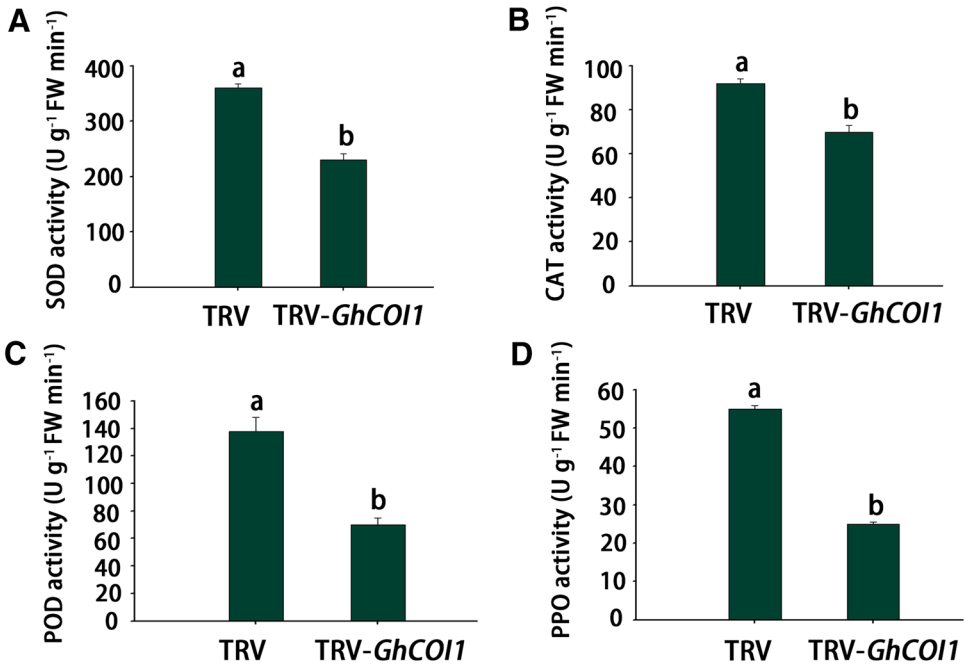

Fig. 8 Effects of *Alternaria brassicicola* inoculation on the activity of defense-related enzyme in the leaves of TRV control and silenced plants. **a** Analysis of SOD activity in the leaves of TRV control and silenced plants. **b** Analysis of CAT activity in the leaves of TRV control and silenced plants. **c** Analysis of POD activity in the leaves of TRV control and silenced plants. **d** Analysis of PPO activity in the leaves of TRV control and silenced plants. The values represent the means, and error bars represent the SEs of three biological replicates of three independent lines. The different letters indicate significant differences ($p < 0.05$; Duncan's multiple range test); the same letters indicate no significant differences

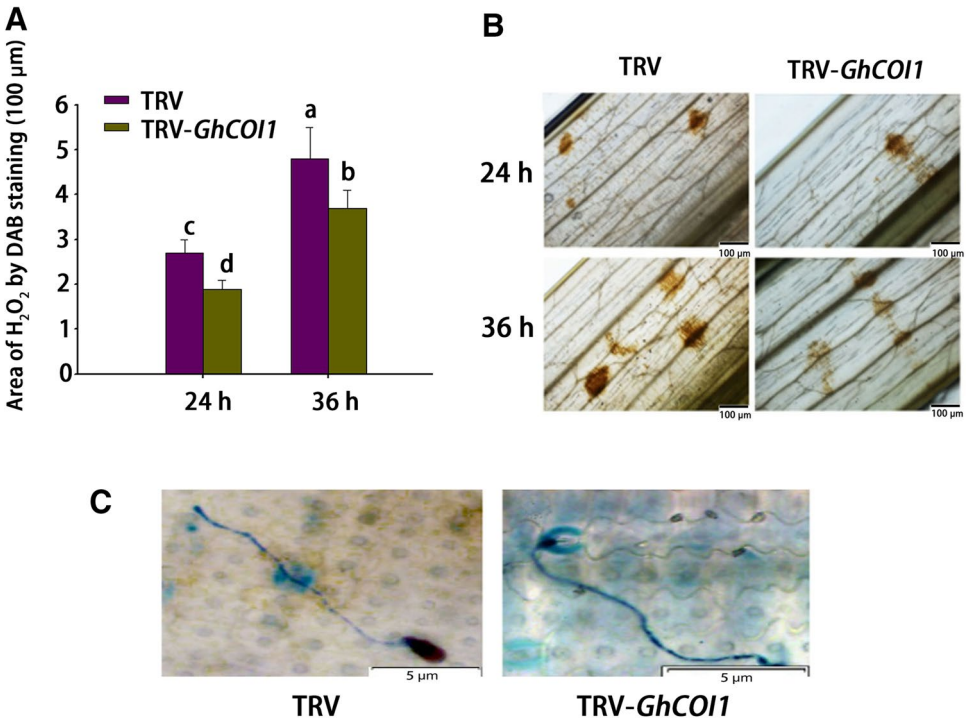

Fig. 9 Effects of *Alternaria brassicicola* inoculation on H_2O_2 accumulation in the leaves of TRV control and silenced plants. **a** The area of H_2O_2 staining was analyzed by DAB staining at 24 h and 36 h after inoculation of the leaves of TRV control and silenced plants. **b** The accumulation of H_2O_2 was measured via DAB staining at 24 h and 36 h after inoculation of the leaves of TRV control and silenced plants. The scale bars indicate 100 μm. **c** Hyphae in leaves inoculated in different ways. In the TRV control plants, the hyphae passed near the stomata and penetrated the leaves directly. In the TRV-*GhCOI1* plants, the hyphae entered the leaves through the stomata. The scale bars indicate 5 μm. The values represent the means, and the error bars represent the SEs of three biological replicates of three independent lines. The different letters indicate significant differences ($p < 0.05$; Duncan's multiple range test); the same letters indicate no significant differences

However, the area of H_2O_2 accumulation was 1.5-fold greater in the leaves of the TRV control plants than in the leaves of the silenced plants. At 36 h, the area of H_2O_2 accumulation in leaves of the TRV control plants was 1.3-fold greater than that in the leaves of silenced plants, indicating that silencing of *GhCOI1* reduced the accumulation of H_2O_2.

The images in Fig. 9b show that the color of the tissue with H_2O_2 accumulation occurring around the stomata was lighter in the leaves of the silenced plants than in leaves of the control plants. We speculate that the poor resistance of the silenced plants to *A. brassicicola* was related to the method of invasion. More H_2O_2 accumulated in the subsidiary cells of the leaves of the TRV control plants than in those of the silenced plants, preventing *A. brassicicola* invasion through stomata and increasing the resistance of the control plants. In contrast, only a small amount of H_2O_2 accumulated in the leaves of the silenced plants. Thus, the fungus could directly enter the tissue through the stomata and cause cell necrosis (Fig. 9c).

On the basis of the results in which *GhCOI1* responded to *A. brassicicola* and in which silencing of *COI1* in *Gladiolus* increased the susceptibility to *A. brassicicola* and impaired the induction of SOD, CAT, PPO and POD, we conclude that the JA signal transduction pathway plays a key role in *Gladiolus* defense against *A. brassicicola* and that GhCOI1 is specifically required for the regulation of JA-mediated defense in response to *A. brassicicola*. Moreover, SOD, CAT, PPO and POD are JA-induced defenses that respond to *A. brassicicola*. SOD, CAT, PPO and POD are mediated by *GhCOI1* (Fig. 10). The *GhCOI1* gene product serves as a receptor of the JA signal and activates the JA signaling pathway, thus increasing the downstream activities of the SOD, CAT, PPO and POD enzymes, which then leads to increased *Gladiolus* resistance against *A. brassicicola* (Fig. 10).

Discussion

In *Arabidopsis*, the F-box motif at the N-terminus of AtCOI1 mediates the interaction between COI1 and ASK1, and the 18 LRR motifs at the C-terminus have specificity for the recognition of target proteins (Jin et al. 2004). The integrity

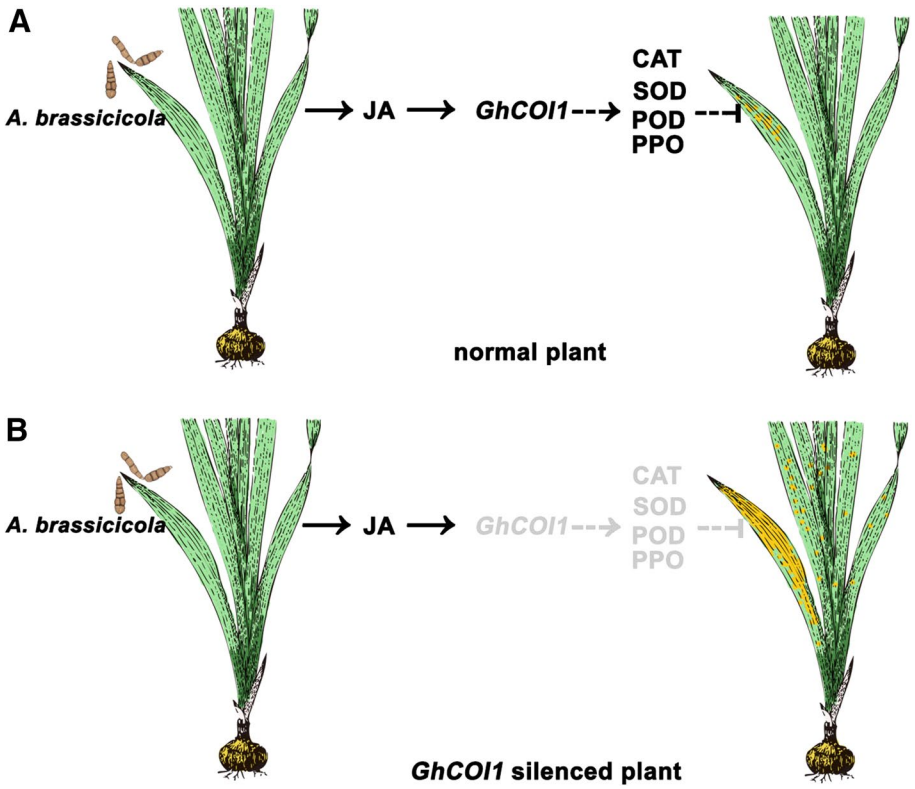

Fig. 10 Diagram showing the involvement of GhCOI1 in response to *Alternaria brassicicola*. **a** When normal *Gladiolus* is invaded by *A. brassicicola*, endogenous JA is induced, which further activates the JA signaling response, including the key regulator *GhCOI1*. GhCOI1 is necessary for the induction of SOD, POD, PPO, and CAT in the *Gladiolus* defense response to *A. brassicicola* infestation. *GhCOI1* also induces H_2O_2 accumulation, blocking the ability of the fungus to directly enter leaf tissues through the stomata. **b** When *A. brassicicola* invades *GhCOI1*-silenced *Gladiolus*, the JA signal transduction is weak and cannot induce a sufficient amount of SOD, POD, PPO and CAT for a defense response. The level of H_2O_2 is relatively low, contributing to the infestation through the stomata. The yellow dots indicate symptoms of *A. brassicicola*. (Color figure online)

of the AtCOI1 protein contributes to its stability in vivo (Yan et al. 2013). In wheat, the predicted TaCOI1 protein has an F-box domain, several LRRs and a conserved AMN1 domain (Liu et al. 2017). In the present study, we found the F-box motif to be present at the N-terminus of the GhCOI1 protein. The GhCOI1 protein also contains 9 LRR motifs and 6 JA-binding sites, consistent with AtCOI1 and TaCOI1 (Fig. 1).

AtCOI1 expression has been detected in the roots, stems, leaves, petals and stamens of *Arabidopsis* (Adie et al. 2007; Benedetti et al. 1998; Melotto et al. 2006). In *Capsicum*, *CaCOI1* is expressed in the roots, stems, leaves and flowers, with the greatest expression occurring in the flowers (Hu et al. 2013). However, the expression of *COI1* in *Gladiolus* differs from that in *Capsicum* and *Arabidopsis*. In our study, the greatest levels of *GhCOI1* mRNA expression were detected in the leaves, followed by the roots; relatively low levels were detected in the flowers, and the lowest levels were detected in the cormels (Fig. 4). In recent years, homologous *COI1* genes have been found in plant species such as rice and wheat. For example, there are three homologous *COI* genes in the rice genome: *OsCOI1a*, *OsCOI1b*, and *OsCOI2* (Lee et al. 2013). Notably, inoculation of *Pseudomonas fluorescens* onto wheat roots induces expression of *COI2* (Okubara et al. 2010). It remains to be explored whether homologous *COI* genes are expressed in other *Gladiolus* tissues or organs.

It has been demonstrated that the COI protein is a key regulator within the JA pathway (Farmer and Ryan 1990). As a signaling component of plant stress resistance, MeJA participates in the host response to pathogenic microbial stress and induces plant disease resistance. The stress resistance induced by JA is closely related to its concentration (Mehari et al. 2015; Cohen and Flescher 2009). A certain concentration of MeJA has a positive effect on the defense response of host plants. When the concentration of MeJA is not suitable, its induction effect decreases rapidly and can even become toxic (Feng et al. 2009). In the present study, we also found that *GhCOI1* is induced by low concentrations of MeJA and repressed by high concentrations of MeJA in *Gladiolus* (Fig. 4); a suitable concentration of MeJA is 0.1 mM.

In this study, *GhCOI1* expression increased sharply during the first 6 h in response to 0.1 mmol/L MeJA treatment but then decreased slightly, after which a high level was maintained (9–72 h) (Fig. 4). A similar expression pattern was also observed in rice and grape (Ye et al. 2012; Wang et al. 2012). We hypothesize that there may be negative feedback or counteraction by other signals to repress the expression of *GhCOI1* after 6 h. It seems that *GhCOI1* is tightly regulated in plants. Thus, it will be interesting to explore the regulatory mechanism. In JA signaling, an alternative splice variant of JAZ10 was shown to exert negative feedback on its own signal transduction (Moreno et al. 2013).

Silencing *GhCOI1* in *Gladiolus* reduced *GhCOI1* transcript levels. Moreover, the fungal biomass within TRV-*GhCOI1* plants was significantly greater than that within control plants, suggesting that the *GhCOI1* gene is involved in the regulation of *Gladiolus* disease resistance. To verify this effect of *GhCOI1*, we used the trypan blue staining method to observe cell necrosis and fungal infection. The results showed that the necrotic area of leaf cells in the TRV-*GhCOI1* plants was significantly greater than that of leaf cells in the TRV control plants at 36 h after inoculation (Fig. 7c). Such increased necrosis was beneficial to the growth of the fungus and improved infectivity. Additionally, the hyphae on the TRV-*GhCOI1* plants were longer than those on the TRV control plants at 12 h after inoculation (Fig. 6c), demonstrating that silencing *GhCOI1* promotes the hyphal growth.

Previous studies have shown that *A. brassicicola* can infect plants directly or through stomata (Sharma et al. 2014), and reactive oxygen species can induce stomatal closure, thereby preventing hyphae from entering plant tissue through stomata. Figure 9b shows that H_2O_2 accumulated around some of the stomata of the TRV control plants, thereby inhibiting fungal invasion. Indeed, trypan blue staining confirmed that in the TRV-*GhCOI1* plants, fungal hyphae were able to enter stomata but that hyphae did not enter the stomata of the TRV control plants; rather the hyphae expanded around these structures (Fig. 9c).

PPO catalyzes the formation of lignin and other phenolic oxides, producing a protective barrier against invading pathogens. CAT, SOD and POD are defensive enzymes that scavenge reactive oxygen species in cells, and inoculation of *Solanum* leaves with *Alternaria solani* was shown to increase phenylalanine ammonia lyase (PAL), POD and PPO activities (Patykowski 2006). In addition, PPO, CAT and SOD activities increase after inoculation of *Brassica* with *A. brassicicola*, and the activities of PPO, SOD and PAL are greater in resistant varieties than in susceptible varieties (Hou and Wang 2008). In the present study, infection by *A. brassicicola* increased the activities of SOD, POD, PPO and CAT. In addition, the POD, PPO and CAT activities were significantly lower in the *GhCOI1*-silenced plants than in the TRV control plants (Fig. 8), suggesting that *COI1* is a positive regulator of the disease resistance process. Silencing of the *GhCOI1* gene thus impairs inducible defenses and increases susceptibility to *A. brassicicola*.

In summary, the *GhCOI1* gene was identified and functionally characterized in this study. The GhCOI1 protein acts as a receptor for JA and is a key regulator of JA signaling. Silencing the *GhCOI1* gene hinders the plant response to *A. brassicicola* and ultimately leads to susceptibility,

which can result in leaf cell death. On the basis of these findings, we will apply genetic engineering techniques in the future to improve the disease resistance of *Gladiolus* and to provide a theoretical and technical foundation for improving the quality of cormels and cut flowers.

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Author contributions SSS and CYW designed the experiments. SSS, CYW and JW performed the experiments. SSS and XHZ analyzed the data. SSS wrote the manuscript. JW, JNH and MFY revised the manuscript.

Compliance with ethical standards

Conflict of interest All authors declare that they have no conflicts of interest.

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