



Overexpression of the chitinase gene *CmCHI* from *Coniothyrium minitans* renders enhanced resistance to *Sclerotinia sclerotiorum* in soybean

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Abstract Pathogenic fungi represent one of the major biotic stresses for soybean production across the world. *Sclerotinia sclerotiorum*, the causal agent of Sclerotinia stem rot, is a devastating fungal pathogen that is responsible for significant yield losses in soybean. In this study, the chitinase gene *CmCHI*, from the mycoparasitic fungus *Coniothyrium minitans*, which infects a range of ascomycetous sclerotia, including *S. sclerotiorum* and *S. minor*, was introduced into soybean. Transgenic plants expressing *CmCHI* showed higher resistance to *S. sclerotiorum* infection, with significantly reduced lesion sizes in both detached stem and leaf assays, compared to the non-transformed control. Increased hydrogen peroxide content and activities of defense-responsive

enzymes, such as peroxidase, superoxide dismutase, phenylalanine ammonia lyase, and polyphenoloxidase were also observed at the infection sites in the transgenic plants inoculated with *S. sclerotiorum*. Consistent with the role of chitinases in inducing downstream defense responses by the release of elicitors, several defense-related genes, such as *GmNPR2*, *GmSGT-1*, *GmRAR1*, *GmPR1*, *GmPR3*, *GmPR12*, *GmPAL*, *GmAOS*, *GmPPO*, were also significantly upregulated in the *CmCHI*-expressing soybean after inoculation. Collectively, our results demonstrate that overexpression of *CmCHI* led to increased accumulation of H₂O₂ and up-regulation of defense-related genes and enzymes, and thus enhanced resistance to *S. sclerotiorum* infection while showing no detrimental effects on growth and development of soybean plants.

Xiangdong Yang and Jing Yang have contributed equally to this work.

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Introduction

Soybean [*Glycine max* (L.) Merr.] provides one of the primary sources of vegetable oil and protein for both human consumption and use in livestock feed. To meet the continually increasing demand for soybean, many challenges must be overcome to increase

soybean production in a sustainable manner. In soybean, more than 30 diseases caused by fungi, bacteria and viruses have been identified. Among these biotic stresses, *Sclerotinia* stem rot (SSR, also known as white mold) caused by the cosmopolitan soil-borne fungal pathogen, *Sclerotinia sclerotiorum*, is the second most damaging disease on soybean yield worldwide and accounts for 1.6 billion kilograms of yield loss each year in the US alone (Ranjan et al. 2019), depending on the incidence and severity of the disease (Peltier et al. 2012). Globally, even as high as 60% of yield reductions were reported in some soybean growing regions when climatic factors such as prolonged cool and wet conditions are conducive to disease development (Cunha et al. 2010). Continuous cultivation and canopy density influenced by changes in cultivation practices, such as row spacing, soil fertility, and irrigation, also greatly contribute to disease severity and prevalence (Workneh and Yang 2000). Despite the existence of several tools for SSR management, such as cultural, chemical, and biological control practices, more effective and sustainable control of SSR still largely relies on the genetic resistance of soybean cultivars. However, no available soybean cultivar is completely resistant to SSR, and only moderately resistant cultivars have been identified in some soybean germplasms until now (Collard and Mackill 2008; Guo et al. 2008; Vuong et al. 2008; Zhao et al. 2015; Mccaghey et al. 2017).

S. sclerotiorum has been viewed as a predominately necrotrophic fungus despite the existence of a brief biotrophic phase in pathogenic development (Bolton et al. 2006; Kabbage et al. 2015). It can secrete a repertoire of pathophysiological factors, including oxalic acid (OA), and cell wall degrading enzymes, such as pectinases, cellulases, and hemicellulases, to facilitate plant cell wall degradation and host colonization. Of these factors, OA is usually considered a key virulence factor in the pathogenic success of *S. sclerotiorum* (Cessna et al. 2000; Dutton and Evans 1996; Godoy et al. 1990; Kabbage et al. 2015; Kim et al. 2008; Rollins 2003; Williams et al. 2011). Moreover, it was reported that other virulence factors targeting host responses may also be involved in the pathogenicity of *S. sclerotiorum* (Pedras and Ahia-honu 2004; Zhu et al. 2013). Many attempts have been made to confer pathogen resistance by introducing heterologous genes of the OA-metabolizing enzymes, such as oxalate oxidase and oxalate decarboxylase

(Cober et al. 2003; Cunha et al. 2010; Dias et al. 2006; Donaldson et al. 2001; Hu et al. 2003; Kumar et al. 2016; Livingstone et al. 2005; Walz et al. 2010; Yang et al. 2019). Silencing of soybean NADPH oxidases (GmRBOHs) has also been shown to enhance resistance to *S. sclerotiorum* (Ranjan et al. 2017). As an important class of antifungal substances in plants and vertebrates, chitinases can inhibit fungal growth by hydrolyzing chitin, which is a major component of the cell wall in most pathogenic fungi and nematodes. They can also release pathogen-borne elicitors, which can further induce downstream defense response in the host plants (Dana et al. 2006; Zhang et al. 2016). Transgenic plants overexpressing chitinases from several origins have been shown to exhibit improved resistance to a wide range of fungal pathogens and even abiotic stresses (Dana et al. 2006; Kumar et al. 2009; Roslan and Anji 2011; Zhang et al. 2016). Enhanced defense responses by the overexpression of chitinase was reported to be mediated by the rapid accumulation of reactive oxygen species (ROS) and peroxidases as well as the increased expression of several defense-related genes in the transgenic plants (Kumar et al. 2009; Zhang et al. 2016).

Coniothyrium minitans is an important mycoparasitic fungus that can parasitize and destroy both hyphae and sclerotia of fungi of the genus *Sclerotinia* (Ren et al. 2007; Sun et al. 2017). *C. minitans* has been widely used as a biological agent to control diseases caused by *S. sclerotiorum* and *S. minor* in many vegetable crops and rapeseed (Sun et al. 2017). The mechanisms underlying the *C. minitans*-mediated biocontrol of *Sclerotinia* include mycoparasitism, degradation of oxalate secreted by *S. sclerotiorum*, and antifungal activity (Lou et al. 2016). As a biological control agent on *S. sclerotiorum* and *S. minor*, *C. minitans* exhibits strong biocontrol activity against a range of plant fungal pathogens. Extracellular lytic enzymes from *C. minitans*, such as chitinase, play an essential role in the penetration of the host cells and facilitation of mycoparasitism of *C. minitans* on *S. sclerotiorum* (Lou et al. 2016).

In this study, we investigated the effectiveness of the *C. minitans* chitinase in improving *S. sclerotiorum* resistance in transgenic plants. A *CmCHI* gene from *C. minitans* was introduced into soybean and transgenic lines were evaluated for their resistance to *S. sclerotiorum*.

Materials and methods

Vector construction and plant transformation

The chitinase-encoding full-length *CmCHI* gene (GenBank accession number AF285086.1) was first isolated from *C. minitans* and kindly provided by Prof. Daohong Jiang of Huazhong Agricultural University, China. Plasmid pTF101-35S, which contains a phosphinothricin acetyltransferase gene (*BAR*) expression cassette conferring glufosinate resistance, was used to express *CmCHI*. For transformation vector construction, the 1.3-kb open reading frame of *CmCHI* was amplified from the plasmid pMD18-*CmCHI* using the primers CmCH1-F1 (5'-GCTCTAGAATGATGC

TCTTTGCTCTTGTATCAATTCTC-3', *Xba*I site underlined) and CmCH1-R1 (5'-GAGAGCTCC-TAGTTGTTGGGGAAGCCAGACC-3', *Sac*I site underlined). The amplified fragment was digested with the endonucleases *Xba*I and *Sac*I, and then placed under the control of the cauliflower mosaic virus (CaMV) 35S promoter and nopaline synthase terminator (Fig. 1a). The resulting construct pTF101-*CmCHI* was introduced into the *Agrobacterium tumefaciens* EHA101. For genetic transformation experiments, soybean cultivar Williams 82 was used and the transformation protocol was performed as described previously (Yang et al. 2019). The regenerated and healthy plants were grown under greenhouse conditions at 25 ± 2 °C and a 16/8 h light/dark cycle for subsequent molecular characterization.

Molecular analysis of transgenic soybean plants

The regenerated plants were first screened by LibertyLink[®] strip (EnviroLogix Inc., USA). The presence of the foreign gene was detected by polymerase chain reaction (PCR) using the specific primers CmCH1-F2/CmCH1-R2 which amplified the *CmCHI* coding sequences (Table S1). Transgenic progenies of subsequent generations (T₁ to T₄) were screened by both glufosinate (1500 mg/L) spraying and PCR with the primers mentioned above. Southern blot analysis was conducted to confirm the integration and insertion copy numbers of the foreign gene. Total genomic DNA was extracted from the young leaves of T₂ transgenic plants, using a modified cetyltrimethylammonium bromide method described by Telzur et al.

(1999). After digestion with *Xba*I, which cut once in the plasmid pTF101-*CmCHI*, DNA fragments were separated by agarose gel electrophoresis and blotted onto the positively charged nylon membrane (GE Amersham, USA). The hybridization probe was obtained by amplifying the 1.3-kb fragment that corresponded to the full open reading frame of *CmCHI* using the primers CmCH1-F2/CmCH1-R2. The membrane was then hybridized by the digoxigenin (DIG)-labeled *CmCHI* probe. Hybridization and subsequent washes were performed at 65 °C using the conditions described in the DIG High Prime DNA Labeling and Detection Starter Kit II (Roche, Germany). Signal visualization was conducted with BCIP/NBT as the substrate at room temperature.

Reverse transcription (RT)-PCR and chitinase activity assays

RT-PCR was used to determine the expression of *CmCHI*. The leaves of T₃ transgenic plants were ground into powder in liquid nitrogen using mortar and pestle. Total RNA was extracted using the EasyPure PlantRNA Kit (Transgen Biotech, Beijing, China) and was reverse transcribed with the TransScript[®] One-Step gDNA Removal and cDNA Synthesis SuperMix (TransGen Biotech), according to the manufacturer's instructions. RT-PCR was performed with specific primers CmCH1-F3/CmCH1-R3 (Table S1), with the constitutively expressed native soybean gene *GmACT6* (GenBank No. NM_001289231) as the internal control. Chitinase activity was determined using a commercial Plant Chitinase ELISA Kit (Shanghai Enzyme-linked Biotechnology Co. Ltd, Shanghai). Leaf samples were frozen in liquid nitrogen and enzyme extracts were prepared as described by Zhang et al. (2016). Chitinase activity assays were carried out in flat-bottom ELISA plates following the manufacturer's instructions. The color development of the enzymatic reaction (horseradish peroxidase and 3,3',5,5'-tetramethylbenzidine) was read at 450 nm with a plate reader (ELx800, BioTek, USA) and chitinase activity (mU/mL) was calculated using the standard curve.

H₂O₂ and defense-related enzyme activity analysis

H₂O₂ content was determined in transgenic plants at 2 and 4 h after inoculation with *S. sclerotiorum*, as

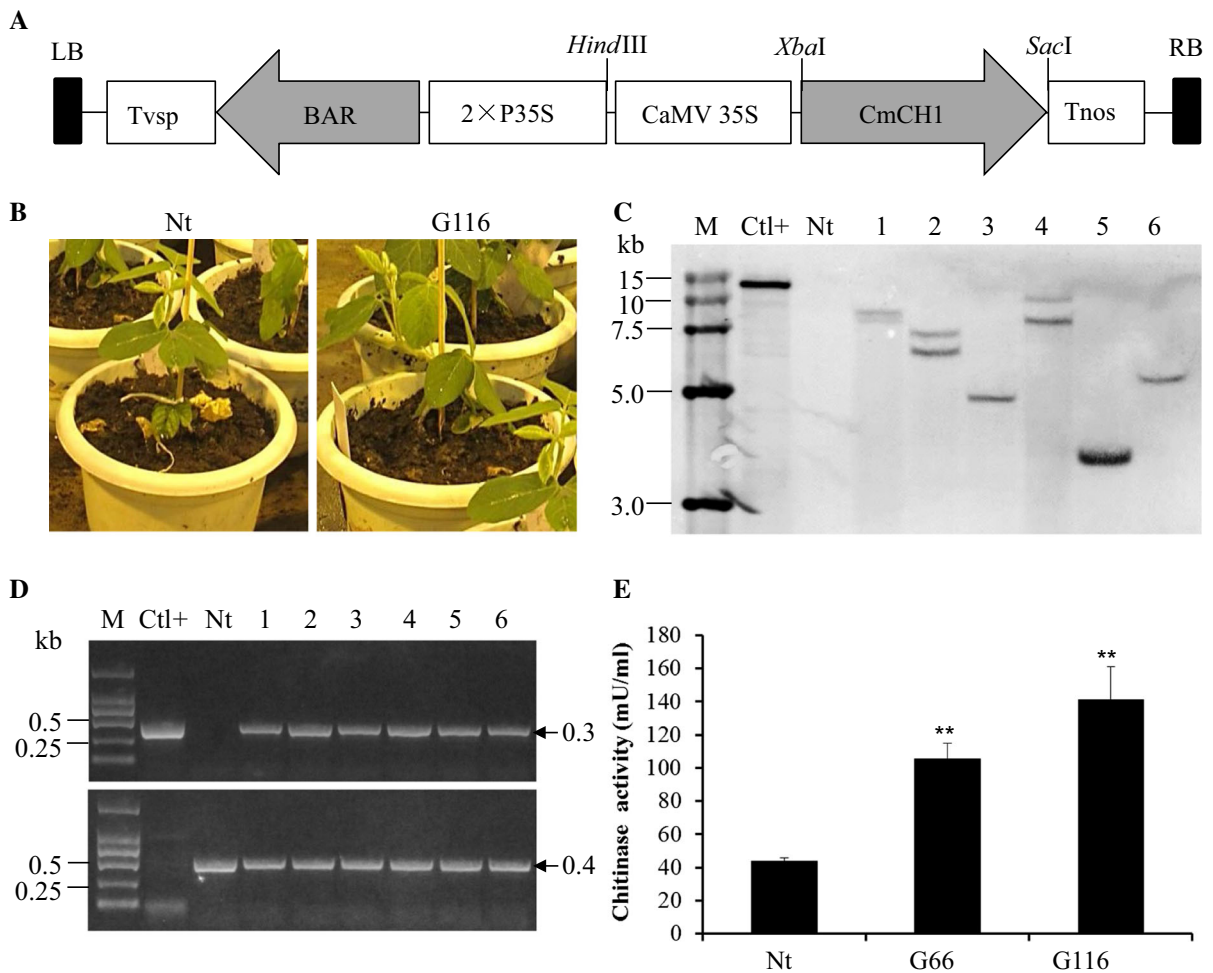


Fig. 1 Generation and characterization of transgenic soybean plants. **a** Schematic diagram of the T-DNA region of the expression vector pTF101-CmCHI for soybean transformation. The selectable marker *BAR* gene confers glufosinate resistance for selection of transgenic plants; LB, left border; RB, right border. **b** T₀ transgenic plants grown in a greenhouse. **c** Southern blot analysis of the T₂ transgenic soybean plants. Genomic DNA samples (~30 µg) were digested with *Xba*I and hybridized against a DIG-labeled *CmCHI* probe. M, DNA marker (15 k);

Ctl+, positive control; Nt, non-transformed plants; G9, G17, G29, G58, G66, G116, T₂ transgenic plants. **d** RT-PCR analysis of transgenic plants (lines 1–6 transgenic plants) and NT control. The soybean *GmACT6* gene was used as an internal control. **e** Chitinase activity analysis. Crude enzyme extracts were prepared from leaves of the transgenic and NT plants. Chitinase activity was analyzed using a commercial Plant Chitinase ELISA Kit, and the absorbance was measured at 450 nm. The asterisks indicate significant differences at $P < 0.01$

described by Kumar et al. (2016). H₂O₂ was extracted and quantified from the leaves of the transgenic and NT plants using the Micro Hydrogen Peroxide Assay Kit (Solarbio, Beijing, China), according to the instructions of the manufacturer. Absorbance was measured at 415 nm, and a standard curve was used to calculate the levels of total H₂O₂ (µmol/g · FW). To investigate the influence of *CmCHI* expression on activities of the defense-related enzymes including phenylalanine ammonia lyase (PAL),

polyphenoloxidase (PPO), peroxidase (POD), and superoxide dismutase (SOD) in transgenic soybean, the crude enzymes were extracted from the leaves of T₃ transgenic and NT plants 4 h after *S. sclerotiorum* inoculation, and enzyme activity was measured as previously reported for PAL, POD, PPO, and SOD (Du et al. 2018). All the samples were analyzed in three biological replicates.

S. sclerotiorum resistance assays

Resistance bioassays of the transgenic lines were performed using detached stems and leaves, as described previously (Yang et al. 2019), using *S. sclerotiorum* collected from an infected soybean field located in Jilin province, China. Sclerotia were grown at room temperature on potato dextrose agar (PDA) and 5-mm mycelial plugs taken from the actively growing edge were used as the inoculum. For the detached stem bioassay, a single mycelial plug was placed on each detached stem from the six-week-old plants and incubated at 25 °C in the dark. Lesion length (cm) on the main stem was measured at 96 h after inoculation. For detached leaf assays, actively growing *S. sclerotiorum* agar plugs were placed on the adaxial surface of the detached leaves and incubated at the conditions described above. Necrotic lesions (cm²) were monitored and photographed 5 d after inoculation as described by Cunha et al. (2010). Six detached stems or leaves were inoculated for each transgenic line tested.

Quantitative RT-PCR analysis of the disease-responsive genes

To analyze the relative transcript levels of defense-related genes in the transgenic soybean plants after *S. sclerotiorum* challenge, quantitative RT-PCR (qRT-PCR) analysis was conducted using gene-specific primers (Table S1). Leaf samples were collected from the T₃ transgenic and NT plants 4 h following inoculation with *S. sclerotiorum*. Total RNA extraction and first-strand cDNA synthesis were carried out as mentioned above. Quantitative RT-PCR was performed with an ABI PRISM 7500 Fast Real-Time PCR System (Applied Biosystems, CA, USA), under the following reaction conditions: 50 °C for 2 min, 95 °C for 10 min, 45 cycles of 95 °C for 2 min, 62 °C for 30 s, and 72 °C for 30 s. The relative transcription levels were calculated using the 2^{ΔΔCt} method with the soybean *GmACT6* gene as a reference. All experiments were conducted with three biological replicates.

Agronomic performance of the transgenic lines

CmCH1 transgenic lines were grown at the experimental station in Jilin province, China, without *S. sclerotiorum* inoculation. At maturity, 20 plants from

each transgenic line were randomly sampled and agronomic traits were measured and recorded.

Statistical analysis

Analysis of variance was used to evaluate the data using SPSS software v.17.0 (SPSS Inc., Chicago, IL, USA). Differences between the average values of samples were compared with Student's *t*-test.

Results and analysis

Generation and characterization of transgenic soybean plants

The *CmCH1* gene was first isolated from *C. minitans* and the predicted protein possesses 397 amino acids with a calculated molecular weight of 48 kDa and a theoretical isoelectric point of 6.08. The Pfam protein family prediction indicated that CmCH1 belongs to the glyco-8 type II chitinase (data not showed). To test the defense potential of the chitinase CmCH1 for the pathogenic fungi, stable transgenic soybean plants expressing the *CmCH1* gene under the CaMV 35S promoter (Fig. 1a) were produced and evaluated for their resistance to *S. sclerotiorum*. After selection and regeneration, a total of 47 glufosinate-resistant plants were obtained from soybean cultivar Williams 82. Positive transgenic soybean plants were first identified by LibertyLink® strip detection. The expected 1.3-kb PCR product, representing the *CmCH1* full coding sequence was observed in the positive plants, whereas no visible amplification was detected in non-transformed plants, confirming the presence of the foreign gene in the regenerated plants (Fig. S1). All transgenic plants were able to flower normally and produce fertile and viable seeds in the greenhouse and exhibited similar phenotypes to the non-transformed (NT) plants (Fig. 1b). For screening of transgenic plants in the subsequent generations, both glufosinate spraying and PCR analysis were conducted to generate homozygous lines. To further investigate the integration and copy numbers of the T-DNA in the genome, Southern blot analysis was carried out using the *CmCH1* gene as a probe. The results showed that hybridization signals appeared in all six selected transgenic plants with all of the bands greater than the expected fragment size of 2.0-kb fragment located between the *Xba*I site and the

right border (Fig. 1c). Moreover, transgenic soybean events had different hybridization patterns and lower copy numbers of the T-DNA insertions. Expression of *CmCHI* at the mRNA level was further examined by RT-PCR analysis with the presence of a single 332-bp amplified fragment in all six transgenic plants and absent in the NT control (Fig. 1d). To detect the functionality of *CmCHI* in the transgenic lines, chitinase activity of the total plant protein extracts was quantified by ELISA analysis. Data showed strong chitinase activity in the transgenic soybean plants were detected corresponding to its own lytic activity, whereas that of control plants was much lower (Fig. 1e).

Enhanced resistance of transgenic lines to *S. sclerotiorum*

Transgenic lines overexpressing *CmCHI* were tested for resistance against *S. sclerotiorum* by detached stem and detached leaf inoculation assays. The reduction in lesion length on stems and lesion areas on the leaves were calculated to assess disease severity caused by *S. sclerotiorum* infection. As can be seen in Fig. 2a, at 96 h following inoculation with *S. sclerotiorum*, the lesion on the stems of the NT plants proceeded aggressively with advanced necrosis and cottony, white mycelia. Whereas transgenic lines exhibited much smaller lesion sizes (15.06–30.27% of those in the NT control) on the detached stems (Fig. 2b). Similar results were also observed in the detached leaf assay. In this case, the untransformed plants showed highly water-soaked lesions across the whole leaves at 5 days after inoculation (Fig. 2c), whereas significantly decreased lesion sizes (43.16–48.47% of that in the NT control) were detected on inoculated leaves of transgenic plants (Fig. 2d). Consistent results seen in the detached stem and detached leaf assays confirmed that *CmCHI* expression significantly improved the resistance of transgenic soybean against *S. sclerotiorum*. Moreover, stable and enhanced resistance in subsequent generations was further observed in subsequent T₄ transgenic lines (Fig. 2b). When no *S. sclerotiorum* inoculation was involved, we did not observe any visible differences in phenotypic or agronomic traits between the *CmCHI* transgenic lines and non-transformed soybean plants (Table 1).

CmCHI transgenic plants showed increased H₂O₂ accumulation and defense responsive enzyme activity

It has previously been reported that chitinases can release elicitors such as reaction oxygen species (ROS) to enhance the plant defense response against pathogens (Dana et al. 2006; Zhang et al. 2016). In this study, we first detected changes in H₂O₂ levels and defense-related enzymes at inoculation sites of the transgenic plants after infection with *S. sclerotiorum*. Our results showed that the H₂O₂ levels were significantly increased in the transgenic plants as compared to the NT controls at 2 and 4 h after inoculation with the pathogen (Fig. 3a). Moreover, we found that *S. sclerotiorum* infection induced significantly higher activities of the four defense-related enzymes in the transgenic plants compared to the NT plants at 4 h after *S. sclerotiorum* inoculation (Fig. 3b–d). Among these four defense-related enzymes, the ROS scavenging enzymes, SOD and POD, may increase tolerance to oxidative stress in transgenic plants to diminish the toxic effects of excessive ROS such as H₂O₂, whereas PAL and PPO may be involved in enhanced resistance against the pathogen. Collectively, these results indicate that the overexpression of *CmCHI* increased H₂O₂ levels and the activities of the defense-related enzymes.

Enhanced expression of defense-responsive genes in transgenic plants after infection

To further explore the role of *CmCHI* overexpression in enhanced soybean resistance to *S. sclerotiorum*, the transcriptional expression of several defense-related genes, including three hypersensitive response (HR)-related genes, four salicylic acid (SA)-dependent genes, and two jasmonic acid (JA)-dependent genes, were investigated by qRT-PCR analysis. The results showed that transcription levels of all three HR-related genes were higher in the transgenic plants than in the NT control at 4 h after *S. sclerotiorum* inoculation (Fig. 4a). Higher expression levels of HR-related genes may be related to the elevated release of H₂O₂, which has been reported to induce a hypersensitive response in host plants. Interestingly, the expression of both SA- and JA-dependent genes were also significantly increased in the transgenic plants relative to the

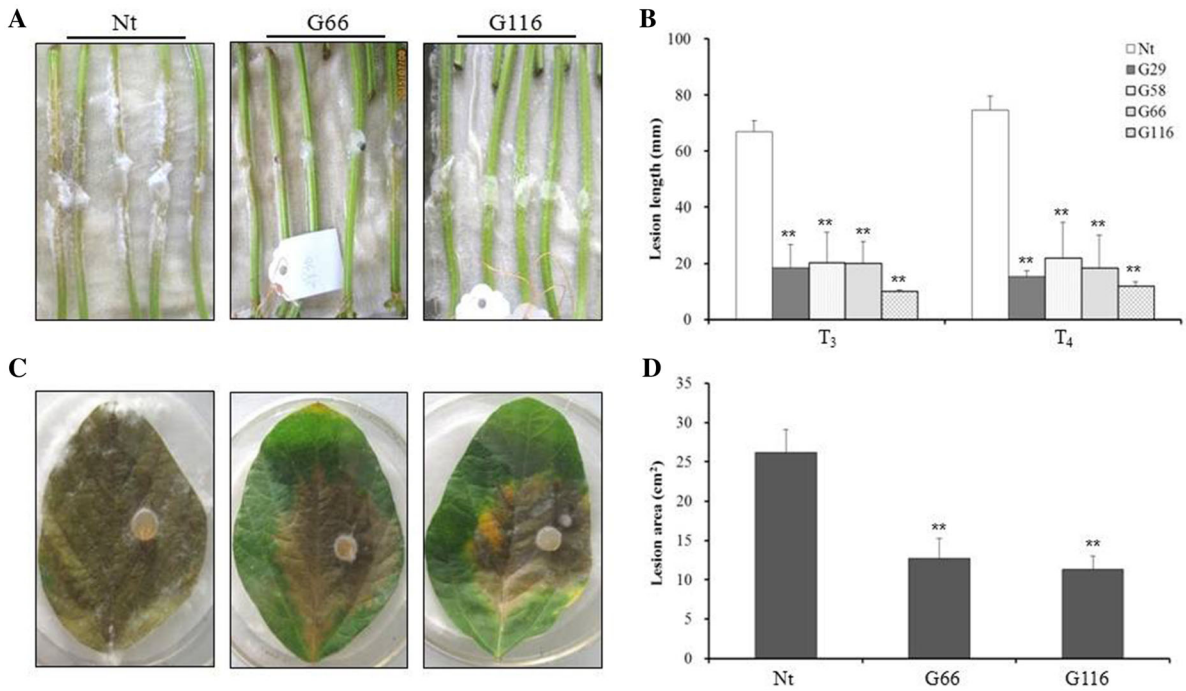


Fig. 2 Overexpression of *CmCHI* enhances soybean resistance to *S. sclerotiorum*. **a** Detached stem assays. Six main stems from six-week-old plants were inoculated and lesion length (cm) on the main stem was measured 96 h after inoculation. **b** Disease resistance evaluation against *S. sclerotiorum* using detached stem assays in T₃ and T₄ *CmCHI* transgenic soybean lines. **c** Detached leaf assays. Six leaves were inoculated from both the

transgenic line and the NT plants and necrotic lesions (cm²) were monitored at 5 d after inoculation. **d** Disease resistance evaluation of T₃ transgenic lines by detached leaf assays. Each experiment was carried out with three independent biological replicates and error bars indicate the standard error. Asterisks indicate a significant difference at *P* < 0.01.

Table 1 Agronomic trait of *CmCHI* transgenic lines in the fields

Agronomic traits	Transgenic events				
	Nt	G29	G58	G66	G116
Leaf shape	Round	Round	Round	Round	Round
Flower color	White	White	White	White	White
Seed coat color	Yellow	Yellow	Yellow	Yellow	Yellow
Hilum color	Black	Black	Black	Black	Black
Maturity period (d)	131	131	131	131	131
Plant height (cm)	95.20 ± 5.59	95.75 ± 7.27	93.14 ± 5.45	94.20 ± 4.78	95.33 ± 6.97
Branching number (/plant)	3.80 ± 1.06	3.25 ± 0.96	3.14 ± 1.46	2.80 ± 1.64	3.00 ± 1.41
Node number (/plant)	21.20 ± 2.36	22.50 ± 1.29	21.00 ± 1.91	21.00 ± 2.39	22.17 ± 0.98
Podding height (cm)	4.80 ± 0.84	4.50 ± 0.58	4.86 ± 0.38	5.20 ± 0.45	4.97 ± 0.41
100-seed weight (g)	16.51 ± 2.03	16.13 ± 1.63	17.16 ± 1.09	17.40 ± 0.85	16.93 ± 1.06

Data were collected from the field station in Jilin province, China, and are presented as the mean ± SD with significant differences at *P* > 0.05.

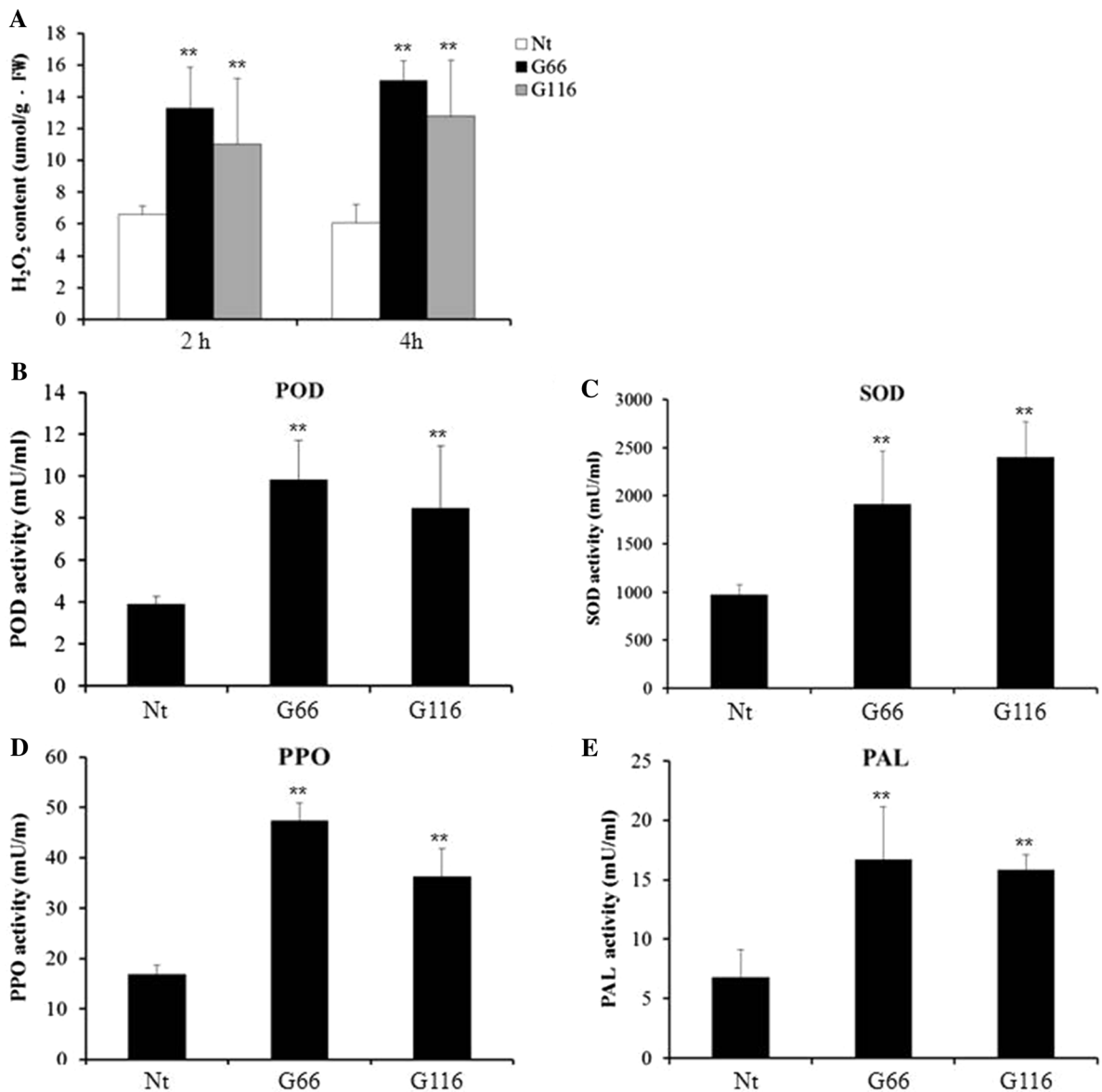


Fig. 3 Increased H₂O₂ levels and defense-responsive enzymes after *S. sclerotiorum* inoculation. **a** Determination of H₂O₂ content in the transgenic soybean plants. At 2 and 4 h after *S. sclerotiorum* inoculation, H₂O₂ was extracted and quantified from the leaves of the transgenic and NT plants and absorbance was read at 415 nm. Increased activities of the defense-responsive enzymes PAL (**b**), PPO (**c**), POD (**d**), and SOD (**e**) in

CmCHI transgenic plants. Fully expanded leaves of 6-week-old soybean plants were inoculated with *S. sclerotiorum* and collected 4 h after infection. Asterisks indicate significant differences between each transgenic line and NT plants at the level of 0.01. The results shown are the mean of three independent experiments

NT plants (Fig. 4b, c), suggesting the involvement of these defense-related genes in *S. sclerotiorum* resistance. Upregulation of defense-related genes such as

GmPPO and *GmPAL* were also consistent with defense responsive enzyme activity analysis (Fig. 3d, e).

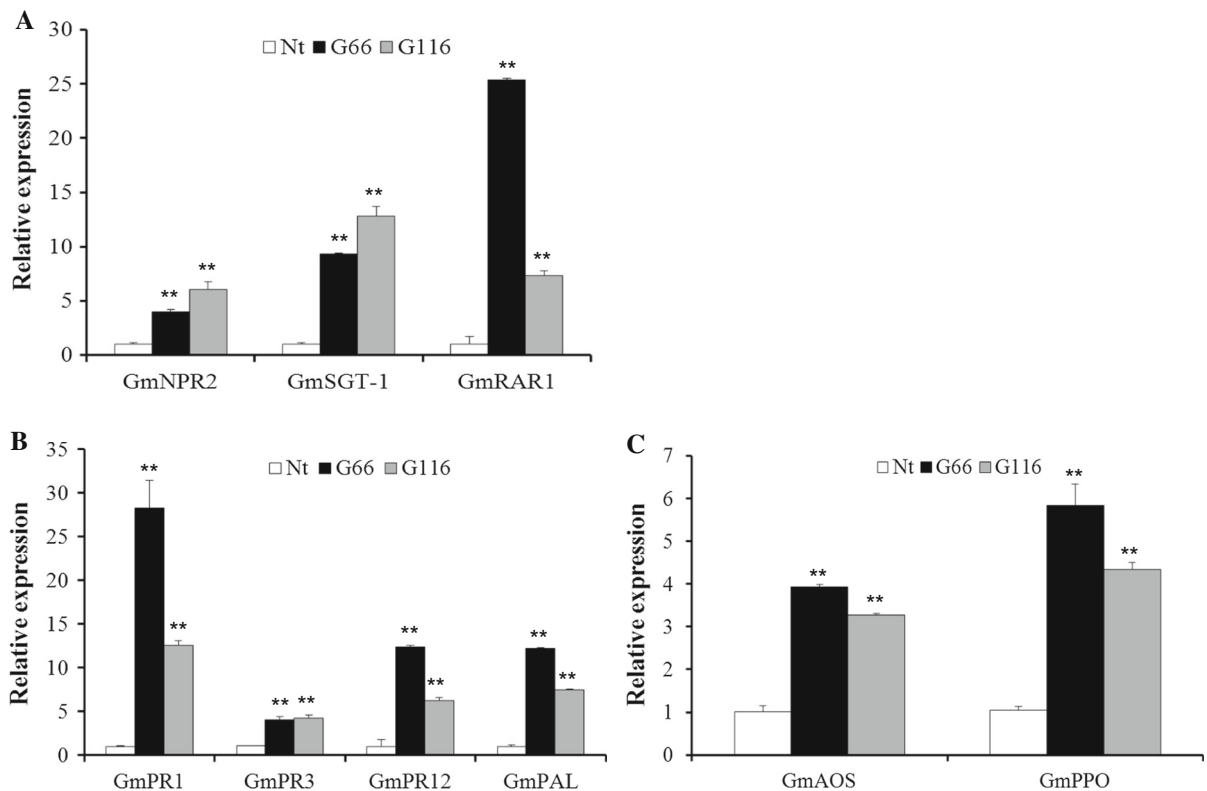


Fig. 4 Relative expression of defense-related genes in *CmCHI* transgenic soybean plants after *S. sclerotiorum* inoculation. Relative expression of three HR-related genes (a), four SA-dependent genes (b), and two JA-dependent genes (c) 4 h after *S. sclerotiorum* inoculation. Relative expression levels were

calculated using the formula $2^{\Delta\Delta Ct}$ relative to the internal control *GmACT6*. Means and standard errors are shown for three independent biological replicates. Asterisks indicate significant differences at the level of 0.01

Discussion

S. sclerotiorum represents one of the major fungal pathogens in many soybean production regions over the world. Traditional breeding for SSR resistance has also been limited by the lack of strong resistance in the soybean germplasm (Cunha et al. 2010; Mccaghey et al. 2017; Yang et al. 2019). Many researchers have attempted to enhance plant protection against pathogens by homologous and heterologous overexpression of disease resistance genes. As a class of antifungal metabolites, chitinases play an important role in plant defense against fungal pathogens by cell wall digestion and releasing pathogen-borne elicitors (Dana et al. 2006; Zhang et al. 2016). Expression of the chitinase genes in plants has proven to be effective in enhancing resistance against a broad range of fungal pathogens (Dana et al. 2006; Zhang et al. 2016). Here, we reported the generation of transgenic soybean plants

overexpressing the gene *CmCHI* from the mycoparasitic fungus *C. minitans*. Our results showed that the transgenic plants exhibited significantly increased resistance to *S. sclerotiorum* as shown in leaf and stem inoculation assay. Compared with the non-transformed control plants, the transgenic plants exhibited a significant reduction in lesion length on the stems and in lesion area on the leaves, which may result from enhanced penetration resistance and limited lesion development of the pathogen at the infection sites. Consistent with enhanced resistance to the pathogen, the chitinase activity of transgenic soybean plants was significantly increased compared to the control plants, suggesting the antimicrobial property of the expressed chitinase in soybean against the pathogen. Similar results were also reported for antifungal activity of chitinase in enhancing resistance against other fungal and bacterial pathogens (Dana et al. 2006; Zhang et al. 2016). We also observed that

the expression of the *CmCHI* exhibited no unexpected influences on the transgenic plants, consistent with the findings that chitinases are not toxic to plant hosts (Dana et al. 2006).

Previous study reported that the chitinases play a dual role, both by inhibiting fungal growth by cell wall digestion and by releasing pathogen-borne elicitors that induce further defense reactions in the host (Dana et al. 2006). The improved resistance against pathogens in plants overexpressing a chitinase gene is not the only consequence of their enhanced activity to decompose chitin and is very likely accompanied by the triggering of other defense-related mechanisms. In the plant and pathogen interaction, rapid generation of ROS is correlated with plant resistance to pathogens and is also one of the most important determinants leading to pathogen penetration failure in invading plant epidermal cells (Yang et al. 2019). However, in pathogenesis of *S. sclerotiorum* on the host plant, the secreted OA can inhibit the oxidative burst of the host plant, thus compromising the H₂O₂-dependent defense responses (Bolton et al. 2010; Cessna et al. 2000; Dutton and Evans 1996; Kumar et al. 2016). In this study, we found that H₂O₂ content was increased in the transgenic plants after *S. sclerotiorum* inoculation, suggesting an H₂O₂-mediated hypersensitive response involved in enhanced resistance to *S. sclerotiorum*. Consistent with this, transcript levels of three HR-related genes (*GmNPR1-2*, *GmSGT1*, and *GmRAR1*) were significantly upregulated in transgenic plants at 4 h after inoculation compared to the NT plants. Moreover, in addition to the upregulation of the HR-related defense genes, a significant upregulation in the expression of several other known defense-responsive genes involved in SA signaling (*GmPRI*, *GmPR3*, *GmPRI2*, and *GmPAL*) and the JA-dependent genes (*GmAOS* and *GmPPO*) were also observed. A rapid induction of SA and JA signaling is associated with an early recognition of the pathogen and involved in resistance in soybean (Ranjan et al. 2019). These results demonstrate that *CmCHI* overexpression increased the accumulation of H₂O₂ and triggered different downstream disease defense-related mechanisms in soybean.

In contrast, although increased H₂O₂ levels could contribute to the enhancement of disease resistance in plants, excessive H₂O₂ is also toxic to plant cells. To maintain the balance of the oxidative state in cells, the ROS scavenging enzymes are also increased to

counteract the toxic effect of excessive H₂O₂. Indeed, the redox buffering capacity of the host is essential to counter the oxidative state imposed by pathogens in host-mediated resistance (Ranjan et al. 2019). In the present study, we found a significant increase in levels of ROS scavenging enzymes, such as POD and SOD, after *S. sclerotiorum* inoculation, which might increase tolerance to oxidative stress in transgenic plants and correlate with the improved resistance to plants pathogens (Dana et al. 2006; Wan et al. 2009). The results are consistent with the findings of Karmakar et al., who observed transgenic plants expressing chitinase and oxalate oxidase 4 genes exhibited elevated H₂O₂ levels and significant increases in activity of ROS scavenging enzymes and reduced membrane damage compared to their wild type counterpart after *Rhizoctonia solani* infection (Karmakar et al. 2016; Zhang et al. 2016).

In this study, *CmCHI*-expressing soybean plants showed enhanced, but not complete, resistance to *S. sclerotiorum* infection. As the likely complex network of responses to the pathogen or its determinants in host plants and multiple factors are involved in the establishment of *S. sclerotiorum*. Among these factors, OA can weaken plant structures and enhance the activity of cell wall-degrading enzymes for the invasion of the pathogen. Overexpression of *CmCHI* can enhance resistance to injury and establishment of fungal infection but cannot completely counteract the effects of OA. It is important to rely on multiple components of defense by pyramiding multiple resistance genes, such as *CmCHI* and other OA-degrading genes, to engineer *S. sclerotiorum* resistance.

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Author contributions YD and QL designed the experiments. XY and JY conducted the experiments and drafted the manuscript. GX, DG, and QZ conducted the *Agrobacterium*-mediated transformation experiments. WX and HL conducted the inoculation assay. LN and YZ participated in the qRT-PCR analyses. All authors participated in the manuscript revision.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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