CGTase, a novel antimicrobial protein from *Bacillus cereus* YUPP-10, suppresses *Verticillium dahliae* and mediates plant defence responses

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**Abstract**

Verticillium wilt is a plant vascular disease caused by the soilborne fungus *Verticillium dahliae* that severely limits cotton production. In a previous study, we screened *Bacillus cereus* YUPP-10, an efficient antagonistic bacterium, to uncover mechanisms for controlling verticillium wilt. Here, we report a novel antimicrobial cyclodextrin glycosyltransferase (CGTase) from YUPP-10. Compared to other CGTases, six different conserved domains were identified, and six mutants were constructed by gene splicing with overlap extension PCR. Functional analysis showed that domain D was important for hydrolysis activity and domains A1 and C were important for inducing disease resistance. Direct effects of recombinant CGTase on *V. dahliae* included reduced mycelial growth, spore germination, spore production, and microsclerotia germination. In addition, CGTase also elicited cotton’s innate defence reactions. Transgenic *Arabidopsis thaliana* lines that overexpress CGTase showed higher resistance to verticillium wilt. Transgenic CGTase *A. thaliana* plants grew faster and resisted disease better. CGTase overexpression enabled a burst of reactive oxygen species production and activated pathogenesis-related gene expression, indicating that the transgenic cotton was better prepared to protect itself from infection. Our work revealed that CGTase could inhibit the growth of *V. dahliae*, activate innate immunity, and play a major role in the biocontrol of fungal pathogens.

**KEYWORDS**

antimicrobial protein, CGTase, induced systemic resistance, *Verticillium dahliae*

1 | **INTRODUCTION**

Cotton (*Gossypium hirsutum*) is one of the most important economic crops and is cultivated in many areas of the world (Ai et al., 2017). Verticillium wilt is a vascular plant disease caused by the soilborne fungus *Verticillium dahliae* (Fadin & Thomma, 2006; Yadeta & Thomma, 2013; Zhang et al., 2019). The pathogen can adhere to the root surface, and its hyphae penetrate roots to colonize the cortex.

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The hyphae then extend into the xylem and form conidia (Daayf, 2015; Zhao et al., 2016). The disease is very difficult to control; there is recent interest in developing alternative methods of control that use biological control agents (Erdogan & Benli-Oglu, 2010). Biological control is an ecologically friendly option for inhibiting the growth of fungal pathogens. It has become common practice to use beneficial antagonistic microorganisms to control soilborne diseases (Goicoechea et al., 2004; Tjamos et al., 2004). Understanding mechanisms employed by biocontrol agents is crucial for isolating effective biocontrol agents and developing biocontrol strategies.

Biological control agents are most often isolated by screening the rhizosphere or endophyte population for organisms that inhibit the growth of a target pathogen in vitro (Brien, 2017). The control agent could provide inhibition by competing for nutrients and by producing multiple antibiotics or polysaccharide-degrading enzymes to control the pathogen or stimulate the growth and immune response of the host (Cai et al., 2013; Card et al., 2009; Druzhinina et al., 2011; Han et al., 2015; Jan et al., 2011; Michelsen & Stougaard, 2012; Michelsen et al., 2015; Santoyo et al., 2012; Sneh, 1998).

Chitin (1,4-2-acetamido-2-deoxy-β-D-glucose), a polymer of β-1,4-glycosidic linked N-acetyl-D-glucosamine units (Flach et al., 1992; Wan et al., 2008), is most commonly associated with the shells or walls of crustaceans, molluscs, algae, and fungi (Somashekar & Joseph, 1996). Chitin plays major roles in fungal cell walls and fungal pathogenicity; fungal pathogens with defective chitin synthesis are significantly less virulent (Soulié et al., 2006). Chitinases can degrade fungal cell wall chitin into small fragments (chitooligosaccharides or chitin oligomers) to limit the development of phytopathogenic fungi (Kim et al., 2017; Ni et al., 2018). Moreover, the fragments can also elicit plant innate immunity against invading pathogens (Kaku et al., 2006; Wan et al., 2008), including a hypersensitive response (HR), oxidative burst, and increased expression of defence-related genes (Cheng et al., 2017). Cyclodextrin glycosyltransferase (CGTase, EC 2.4.1.19) is a carbohydrate-active enzyme that belongs to glycosyl hydrolase family 13. CGTase can catalyse hydrolysis reactions, cleaving the linkages in the starch molecule (van der Veen et al., 2000). Up to the present, more than 50 different CGTase crystal structures have been published; these three-dimensional structures provide a basis for further research on the mechanism and direct molecular engineering of CGTases (Han et al., 2014). Cyclodextrin has a broad range of applications in the food (Samperio et al., 2010), agriculture (Campos et al., 2018), and pharmaceutical industries (Qiu et al., 2014) and environmental engineering (Xu et al., 2019). However, CGTase has not been reported in the field of biological control.

In a previous study, Bacillus cereus YUPP-10, a cotton endophytic bacterium that can hydrolyse polysaccharides at β-1,4-glycosidic linkages, was isolated from enrichment medium using glucomannan as its carbon source. The endophytic bacterium inhibited the growth of V. dahliae, promoted cotton growth, and induced cotton’s immune response against verticillium wilt (Zhou et al., 2017). In this study, a substance capable of degrading β-1,4-glycosidic bonds, CGTase, was selected from B. cereus YUPP-10 by a constructed fosmid library. We discovered that CGTase has antimicrobial activity and induces resistance. In addition, we have revealed the key domains responsible for its hydrolytic activity and resistance induction. Further experiments demonstrated that CGTase is a potential functional protein for improving cotton resistance to verticillium wilt.

### RESULTS

#### 2.1 CGTase cloning and sequence analysis

A 2,157 bp open reading frame was cloned from a fosmid library. The BLAST results indicated that this gene encodes a CGTase (Figures 1a and S1a) that includes 718 amino acids, about 75 kDa.

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**FIGURE 1** Identification, analysis, and predicted domains of CGTase. (a) Phylogenetic analysis of CGTase and its orthologs from other bacteria. The neighbour-joining tree was constructed using the MEGA 7 program. (b) Sequence alignment of CGTase in this study with α-, β-, and γ-CGTase in other reports. There are six different domains in the CGTases (A1, A2, C, D, and E). (c) Predicted conserved domains were determined by online SMART (http://smart.embl-heidelberg.de/smart/set_mode.cgi?GENOMIC=1). CGTase has a N-terminal signal peptide, an α-amylase (catalytic domain), an α-amylase C (C-terminal all-β domain), a TIG (IPT/TIG domain), and a CBM_20 (starch-binding domain).
ZHOU et al. (Figure S1b), an N-terminal signal peptide, and canonical domains including α-amylase (catalytic domain), α-amylase C (C-terminal all-β domain), IPT/TIG (immunoglobulin, plexin, transcription factor-like/transcription factor immunoglobulin) domain, and CBM_20 (starch-binding domain; Figure 1c). The six structural domains (A1, A2, B, C, D, and E; Rahman et al., 2006) were identified in this CGTase from comparisons with other CGTase sequences (Figure 1b). Four highly conserved N-terminal α-amylase catalytic domains and six other functional domains have been marked in Figure 1b.

2.2 CGTase hydrolysis and HR-inducing activity

To test the activity of mutants, the effects of added enzyme on the diameter of transparent circles of carboxymethyl cellulose and glucomannan were measured. The results showed that mutating the six domains reduced hydrolytic activity and the hydrolytic activity of the domain D mutant was the lowest (Figure 2c). The chitinase activity of the CGTase and the six mutants was measured using chitin as a substrate. For the CGTase, A1, B, A2, C, D, and E mutants, the chitinase activities measured with a detection kit were $3.54 \pm 0.04, 3.37 \pm 0.04, 2.77 \pm 0.18, 3.30 \pm 0.21, 2.68 \pm 0.13, 2.11 \pm 0.06,$ and $2.47 \pm 0.13$ U/ml, respectively. These results showed significantly reduced chitinase activity for the domain D mutant (Figure 2d,f). In addition, the concentration of N-acetylglucosamine in reactions with CGTase, A1, B, A2, C, D, and E mutants was $95.79 \pm 2.57, 79.88 \pm 0.62, 76.44 \pm 1.86, 77.68 \pm 1.33, 72.52 \pm 2.47, 57.33 \pm 1.75,$ and $72.02 \pm 7.31$ ng/ml, respectively, showing that the activity decreased the most with the domain D mutant (Figures 2e,f and S2). These data strongly suggest that domain D is the key domain for hydrolytic activity. The elicitor activity of mutants was tested by treating tobacco leaves. The results showed that the A1 and C domain mutants did not induce HR and reactive oxygen species (ROS; Figure 2g,h), indicating these are the key domains for inducing resistance.

2.3 CGTase effectively inhibits the growth of V. dahliae in vitro

The ability of CGTase to inhibit V. dahliae mycelial growth, spore and microsclerotia germination, and spore production were tested. Experiments for each treatment were performed on three independent samples. The results showed that pretreatment of mycelia with CGTase inhibited fungal growth and development (Figure 3a), with higher concentrations of CGTase producing greater inhibition effects. Exposing fungi to 0.48 mg/ml CGTase reduced spore germination, spore production, and microsclerotia germination by 63.58%, 58.44%, and 28.35%, respectively (Table 1). Using visible and scanning electron microscopes, we observed changes in the mycelial morphology such as terminal enlargement (the red

FIGURE 2 Hydrolytic activity and induced resistance of CGTase. (a) CGTase hydrolysed carboxymethyl cellulose and glucomannan, the transparent circles were observed by iodine-KI staining solution. (b) The structural formula of carboxymethyl cellulose and glucomannan, and the cleavage site of carboxymethyl cellulose and glucomannan by CGTase. (c) The diameter of transparent circles in carboxymethyl cellulose and glucomannan treated with CGTase and its mutants. (d) Chitinase activity of CGTase and its mutants by a chitinase activity detection kit. (e) The concentration of N-acetylglucosamine in reactions with CGTase, A1, B, A2, C, D, and E mutants was $95.79 \pm 2.57, 79.88 \pm 0.62, 76.44 \pm 1.86, 77.68 \pm 1.33, 72.52 \pm 2.47, 57.33 \pm 1.75,$ and $72.02 \pm 7.31$ ng/ml, respectively, showing that the activity decreased the most with the domain D mutant (Figures 2e,f and S2). These data strongly suggest that domain D is the key domain for hydrolytic activity. The elicitor activity of mutants was tested by treating tobacco leaves. The results showed that the A1 and C domain mutants did not induce HR and reactive oxygen species (ROS; Figure 2g,h), indicating these are the key domains for inducing resistance.
2.4 CGTase elicits cotton defence against *V. dahliae*

To determine the optimum concentration of CGTase protein for inducing plant immune responses, purified CGTase was serially diluted and infiltrated into the rosette leaves of *Arabidopsis thaliana*. No visible lesions formed 24 hr postinoculation (hpi) in response to injections of 1 or 5 μg/ml CGTase, but sunken lesions that are typical of HR and ROS were visible when concentrations at 10 μg/ml and above were injected (Figure 4a). On the basis of these HR and ROS results, a concentration of 20 μg/ml was selected for subsequent experiments.

Cotton plants were treated with CGTase introduced by injection and root irrigation, and then inoculated with *V. dahliae*. The disease indices of plant treatment by injection and root irrigation were 24.8 and 35.4, respectively, while the disease index of the control plants (protein buffer solution) was 59.5 (Figure 4b,c), showing that the use of CGTase reduced disease by 58.25% and 40.55%, respectively.
Accumulation of callose was visualized by staining with aniline blue (Figure 5a). Compared to control plants, an increased density of callose depositions (number per cm²) was seen in plants treated with CGTase. Jimian 11 cotton roots were treated with CGTase and \textit{V. dahliae}, and the expression of defence and pathogenesis-related (PR) genes in the leaves was investigated using quantitative reverse transcription PCR (RT-qPCR). The highest level of PAL expression was at 48 hr postinoculation (hpi) and was 7.33 times greater than that for the untreated control. Expression levels of PAL at 36 and 60 hpi, POD at 24 hpi, and HSR203J at 24 hpi in treated plants were slightly lower than those of the untreated control, but these differences were not statistically significant. The highest expression level of POD occurred at 12 hpi and was 1.45 times greater than that of the untreated control. Similarly, CGTase treatment also induced \textit{PR1} and \textit{HSR203J} gene expression with the highest levels at 72 hpi that were, respectively, 7.69 and 4.56 times greater than those in the control. \textit{Jaz1} was down-regulated by CGTase treatment at 36 and 60 hpi, but up-regulated at 72 hpi, with an expression level that was 3.61 greater than that of the untreated control (Figure 5b).

2.6 Overexpressing CGTase increased the biomass of \textit{Arabidopsis} seedlings

The effect of CGTase overexpression was studied in \textit{A. thaliana}. Homozygous CGTase-overexpressing transgenic lines were selected and confirmed using a variety of methods, including kanamycin selection and screening with PCR and RT-qPCR (Figure S3). The three lines with the highest CGTase expression were selected for further study. Transgenic plants grew significantly better than wild-type (WT) plants in half-strength Murashige and Skoog (1/2 × MS) medium and nutrient soil (Figure 6a,b). The fresh weight and leaf length of 1-week-old \textit{A. thaliana} plants were measured. Compared to WT, the transgenic lines increased fresh weight and leaf length by 0.6 g and 2.3 cm, respectively (Figure 6c,d).
Overexpression of CGTase enhances Arabidopsis resistance to V. dahliae

The resistance of CGTase-overexpressing transgenic plants to V. dahliae was assessed with an in vitro technique. The transgenic A. thaliana plants were more resistant to verticillium wilt than the WT plants. All WT plants were dead within 2 weeks of inoculation with V. dahliae, while transgenic plants were still alive (Figure 7a). This confirms that transgenic A. thaliana plants that overexpress CGTase were more resistant to verticillium wilt than WT plants.

Greenhouse-grown transgenic plants were noticeably more resistant to the pathogen than WT plants (Figure 7b). The percentage of diseased plants and disease index of the transgenic plants were 34.5% and 27.5, respectively, which were lower than those of the WT plants (Figure 7c,d).

To explore the mechanism of transgenic A. thaliana resistance to V. dahliae infection and colonization, the attachment of pathogenic fungus
to the roots was observed using optical and scanning electron microscopy. It was observed that the root tissue of WT plants was severely damaged by mycelial invasion and propagation (Figure 8a). With transgenic *A. thaliana*, mycelial attachment to the root surface was still observed, but the root damage was not serious (Figure 8a). This indicated that the transgenic *A. thaliana* had the ability to resist infection. With optical microscopy, mycelial attachment and browning of vascular bundles were observed for WT plants. For transgenic *A. thaliana*, although mycelial attachment on the root surface was observed, the amount of mycelial attachment was very small and only part of the vascular bundles browned (Figure 8b). This shows that transgenic *A. thaliana* had the ability to resist infection.

To observe *V. dahliae* colonization with a confocal microscope, *A. thaliana* was infected with *V. dahliae* containing a green fluorescent protein (GFP) tag (Vd592-GFP). We found *V. dahliae* colonization was significantly greater in WT *A. thaliana* than in the CGTase-transgenic plants (Figure 8c). The fungal recovery from stem sections of transgenic plants was markedly lower than that of WT plants (Figure 8d). Dark blue veins and large stained mesophyll areas were observed in WT plants, while light blue veins and mesophyll that was not extensively stained were observed in transgenic plants (Figure 8f). The results show that overexpression of CGTase conferred *A. thaliana* with increased disease resistance against *V. dahliae*.

### 2.8 Overexpression of CGTase activated plant defence

The xylem of *A. thaliana* was observed in longitudinal cuttings of hypocotyl incubated in 10% phloroglucinol. A noticeable increase in the thickness of xylem in transgenic plants was observed (Figure 9a). To explore whether transgenic *A. thaliana* resisted disease by activating ROS, 3,3′-diaminobenzidine (DAB) staining was performed on the leaves. The results showed that there was no significant difference between WT and 35S::CGTase leaves in the absence of pathogen. After pathogen inoculation, both lines showed induced ROS, but the ROS change in transgenic *A. thaliana* was stronger (Figure 9b).

RT-qPCR was used to detect the expression of defence-related genes in *A. thaliana*. CHI, PDF1.2, PR1, and PR2 play important roles in plant disease resistance. In this study, their expression in transgenic *A. thaliana* was significantly higher than that of WT during the critical period of disease resistance, which indicated that the expression of defence genes was more strongly activated in the CGTase transgenic than in WT (Figure 9c).

### 3 DISCUSSION

CGTase is an important industrial enzyme that can transfer starch to glycosyl groups to form cyclodextrin (Li et al., 2014c; Qi et al., 2007). CGTase has been found in many bacteria, including *Bacillus* spp. (Chen et al., 2018; Gimenez et al., 2019; Yap et al., 2010) and *Paenibacillus* spp. (Castillo et al., 2018). CGTase can catalyse four reactions: cyclization, coupling, disproportionation, and hydrolysis (Li et al., 2014b), though the hydrolysis activity is relatively weak (Costa et al., 2009). CGTase is a member of glycoside hydrolase family 13 (GH13) (Buchholz & Seibel, 2008; Stam et al., 2006). In this study, the CGTase could hydrolyse chitin, glucosaminan, and sodium carboxymethylcellulose. Members of this family share a conserved active site architecture and four short conserved sequence regions embedded in a triosephosphate isomerase (TIM) (β/α) 8 structural
fold (Janěček, 1997). Most family members hydrolyse α-glucosidic linkages (McCarter & Withers, 1994), except YUPP-5, a CGTase from Paenibacillus azotofixans that can hydrolyse β-1,4-polysaccharide linkages (Zhou et al., 2012). The CGTase contains α-amylase, α-amylase C, TIG, and carbohydrate-binding module (CBM)_20 active sites. Using chitin as a substrate, we showed that CGTase can hydrolyse β-1,4-linkages to produce N-acetyl-d-glucosamine, which was detected with mass spectrometry. Chitinase and β-1,3-glucanase can degrade chitin in the fungal cell wall and destroy the fungal cytoskeleton to thereby inhibit the pathogenicity and growth of fungi (Fujimori et al., 2016; Sousa et al., 2019). In addition, a chitin-binding site (GDQVS) (Svitil & Kirchman, 1998) was found in the CGTase, showing that CGTase can bind chitin. Findings from this study suggest that the CGTase and chitinase have similar functions. The fungal cell wall is pivotal for maintaining cell shape and function, and helping the fungus meet a variety of challenges so a major fungal disease control strategy is to destroy the cell wall of the fungus. Depending on the species, fungal walls comprise different proportions of β-glucans, chitin, proteins, and cellulose (β-1,3-glucans, β-1,6-glucans, and, in some species, β-[1,3,1,4]-glucans) (Samalova et al., 2017). Chitinase predominantly acts on chitin, while CGTase can hydrolyse many components of the cell wall, so its activities are more diverse.

CGTases comprise six domains (Klein & Schulz, 1991; Lawson et al., 1994). The A1, A2, and B domains form the substrate binding groove and contain the catalytic residues; the C and E domains assist with starch binding (Chang et al., 1998; Penninga et al., 1996); the function of domain D is unknown. Six different CGTase sequences that are conserved with α-, β-, and γ-CGTases were mutated, and then their hydrolysis and HR activities were compared. Our results indicate that domain D is the key domain for CGTase hydrolysis activity and domains A1 and C are the key domains for inducing plant resistance. The functions of CGTase were changed by replacing amino acid residues in some domains (Leemhuis et al., 2010). Improving hydrolysis activity to transfer CGTase to a hydrolytic enzyme by site-directed mutagenesis

**FIGURE 7** Enhanced disease resistance of Arabidopsis plants overexpressing CGTase. Symptoms of wild-type (WT) and overexpressing CGTase plants in 1/2 × Murashige and Skoog medium at 2 weeks (a) and in nutrient soil at 3 weeks (b) after inoculation with Verticillium dahliae. In (b), the overexpressing CGTase plants are in the columns to the left of each red line and the WT plants are to the right of each red line. The disease index (c) and disease plant rate (d) of WT and transgenic plants. Error bars indicate the SD of three biological replicates. Different uppercase letters indicate significant difference at $p < .01$.
has attracted much attention (Fujiwara et al., 1992; Kelly et al., 2007; Nakamura et al., 1994; van der Veen et al., 2001). This indicated that CGTase has a good application prospect. Cotton treated with the CGTase showed improved *V. dahliae* disease resistance, indicating that the CGTase can induce an immune response. Induced systemic resistance is a plant defence response that induces factors, including hydrolase, flagellum, antibiotics, *N*-acyl homoserine lactone, salicylic acid (SA), jasmonic acid (JA), iron carrier, volatiles, and lipopolysaccharide, that correlate with higher pathogen resistance (Bordiec et al., 2011; Cheng et al., 2017; Gamalero et al., 2016; Kloepper & Ryu, 2006; van Loon et al., 2008). HR is involved in the initiation of resistance genes and associated with death-associated signals (Mur et al., 2008), so HR is part of plant innate immunity (Atkinson et al., 1990). HSR203J gene expression is considered a marker of HR (Lee & Nürnberger, 2001; Takahashi et al., 2010; Zhang et al., 2016). ROS is one of the earliest events in HR and a signal that activates downstream cellular processes (Lamb & Dixon, 1997; Vellosillo et al., 2010). In this study, ROS was observed in all CGTase-treated plants, with CGTase treatment causing leaf necrosis that was visible with the naked eye in *A. thaliana* and tobacco leaves. Although no obvious necrosis area was found on cotton leaves, HR marker gene expression was significantly higher in CGTase-treated plants than in control plants. The results showed that CGTase could induce a ROS burst and signal transduction in plants to stimulate plant immunity responses.

Beneficial substances induce systemic defence responses through signalling networks that are controlled by plant hormones like SA, JA, and ethylene (ET) (Hammond-Kosack & Parker, 2003). Some evidence has shown that the SA, JA, and ET pathways crosstalk to adjust the plant defence response for different pathogens. In cotton, *GhPR1* and *GhJaz1* are marker genes for the SA (Zhang et al., 2016) and JA/ET signalling pathways, respectively. In this study, *PR1* expression was activated at 36 hpi, which is within the typical temporal window for plant defence priming. However, *Jaz1* expression was down-regulated at 36 hpi. Thus, our work revealed that CGTase may simultaneously activate the SA pathway while inhibiting the JA pathway. In *A. thaliana*, *plant defensin 1.2* (*PDF1.2*) is a marker gene for the JA/ET signalling pathway (Guo & Stotz, 2007), and the pathogenesis-related *PR1* and *PR2* genes are marker genes for the SA signalling pathway (Glazebrook et al., 2010). Our RT-qPCR results showed that CGTase may be involved in SA and JA signalling pathways in *A. thaliana*.

As defence enzymes, phenylalanine ammonia-lyase (PAL) and peroxidase (POD) can catalyse or participate in the synthesis of plant lignin and phenolic compounds that are involved in the formation of barriers against pathogens (Appel, 1993; Mandal & Mitra, 2007).
Lignification is a physical mechanism of plant disease resistance and is also important for plant growth and development (Barros et al., 2015; Bhuiyan et al., 2009). In this study, the expression of PAL and POD was up-regulated in CGTase-treated cotton. CGTase-dependent effects on cotton callose and A. thaliana xylem were also observed.

In this study, we investigated a novel antimicrobial protein CGTase that can break fungal cell walls (chitin). Furthermore, CGTase also acts as an elicitor protein to induce typical HR, activate early defence-related events, and induce defence and pathogenesis-related genes to resist V. dahliae infection of cotton. A. thaliana is an excellent tool for identifying traits involved in V. dahliae biocontrol (Maldonado-González et al., 2015). Both the growth and disease resistance of transgenic A. thaliana that overexpressed CGTase were enhanced, therefore CGTase may be useful for breeding disease-resistant plants.

4 | EXPERIMENTAL PROCEDURES

4.1 | Fungal strain, plant lines, and culture conditions

The virulent, defoliating V. dahliae strain Vd080 was used in this study. Vd080 was grown on potato dextrose agar (PDA) at 25 °C in the dark.

Nicotiana benthamiana, A. thaliana (Col-0), and Jimian 11, an upland cotton (Gossypium hirsutum) cultivar that is susceptible to V. dahliae, were grown in a greenhouse with a 16/8 hr photoperiod at 22–28 °C.

4.2 | CGTase cloning, mutation, and phylogenetic analysis

B. cereus YUPP-10 was cultured overnight in Luria-Bertani (LB) broth at 37 °C. Genomic DNA was extracted with the Bacterial DNA Kit (Omega). The fosmid library was constructed with the CopyControl Fosmid Library Production Kit (Lucigen). Purified genomic DNA was sheared to approximately 40-kb fragments, isolated, and purified to the desired size range by low melting point agarose gel electrophoresis, then blunt-end ligated to the Cloning-Ready CopyControl pCC1FOS vector. The ligated DNA was packaged and plated on Escherichia coli EPI300-T1², and clones were grown overnight. The CopyControl fosmid clones of
interest were picked and induced to high-copy number using the CopyControl fosmid autoinduction solution. To screen for the target gene, transformants were inoculated onto the same position of two Petri dishes that contained medium prepared as reported by Zhou et al. (2012). After 16 hr at 37 °C, one dish was stained with a 0.2%:2% iodine:KI solution. Transparent colonies were chosen and cultured. Plasmid containing the target gene was extracted and partially digested with Sau3AI. The 5 kb pET28a plasmid fragments were collected and transformed into E. coli DH5α. A transformant displaying glucomannan-degrading activity was selected for sequencing and analysis.

CGTase amino acid sequences were aligned using the National Center for Biotechnology Information online BLAST tool. The phylogenetic tree was generated with MEGA v. 7.0 software (http://megasoftware.net) using the neighbour-joining method with 1,000 bootstrap replications.

Predicted conserved domains were determined using SMART (http://smart.embl-heidelberg.de.smart SET_mode.cgi?GENOMIC) and enzymes were modified with over-expression PCR. The mutation fragments were amplified from pDP66K, which carries α-CGTase.

4.3 | CGTase expression and purification

Signal peptides and the hydrophilicity of protein sequences were forecasted with online tools. Hexahistidine and small ubiquitin-like modifier tags that did not include signal peptides were linked to the N-terminus of CGTase using gene splicing with overlap extension PCR. The PCR products were inserted into Ndel/NotI restriction sites of pET28a. The CGTase encoding region and six other conserved domains were mutated, and mutated genes were cloned into the pET28a vectors that were expressed in E. coli BL21. After purification, recombinant HIS-CGTase presented as a single protein band in SDS-PAGE. The recombinant plasmid was cultivated under optimum conditions, so that a large amount of CGTase was purified by an AKTA Explorer system (Amersham Biosciences). Purified protein concentrations were measured with a microspectrophotometer (Thermo Fisher Scientific) and a BCA protein assay kit (Boster).

4.4 | Antimicrobial activity

V. dahliae Vd080 was grown for 1 week on potato dextrose agar (PDA) at 25 °C in the dark. Then, a colony of Vd080 was grown at 25 °C with shaking (180 rpm) in Czapek liquid medium (30 g/L sucrose, 2 g/L NaNO3, 0.5 g/L MgSO4·7H2O, 0.5 g/L KCl, 0.02 g/L FeSO4·7H2O, and 1.31 g/L KH2PO4). After 6 days, the culture was filtered with four-layer sterile gauze to produce the spore suspension. Two hundred microlitres of spore suspension was coated onto PDA in a Petri dish, and 200 μl of CGTase was added to a 5 mm hole. The same volume of buffer (50 mM Tris.HCl, 150 mM NaCl, pH 7.5) was added in the control treatment. Petri dishes were kept in a dark incubator at 25 °C for 3–5 days before analysis of inhibition zones.

Different concentrations of CGTase (0.12, 0.24, and 0.48 mg/ml) were mixed with equal volumes of 2 × 103 cfu/ml Vd080 spore suspension to make 600 μl mixtures. Then, 20 μl of the mixture was added as a hanging drop to the glass slide and cultured at 25 °C for 3 hr. The germination rate of conidia was measured with an optical microscope. Another 500 μl of the mixture was grown for 2 days at 25 °C with 180 rpm shaking, and then conidial sporulation was quantified with an optical microscope. Each experiment was repeated three times.

Vd080 microsclerotia were produced following a procedure that uses modified basal agar medium (5 g/L glucose, 0.2 g/L NaNO3, 0.52 g/L KCl, 0.52 g/L MgSO4·7H2O, 1.52 g/L KH2PO4, 3 μM thiamine.HCl, 0.1 μM biotin) (Hu et al., 2013; López-Escudero et al., 2007). Different concentrations of CGTase (0.12, 0.24, and 0.48 mg/ml) were mixed with equal volumes of microsclerotia to make 100 μl mixtures. The mixture was grown for 24 hr at 25 °C with 180 rpm shaking. The germ tubes of 100 microsclerotia were assayed with an optical microscope. The evaluation standard for sclerotal germination was that the length of the germ tube exceeded the radius of the microsclerotia. Each experiment was repeated three times.

4.5 | Hydrolytic activity

A chitinase activity detection kit (Solarbio) was used to detect the chitin hydrolysis activity of WT and mutant CGTases. The hydrolysates of chitin were also analysed with liquid chromatography mass spectrometry (LC-MS). The diameters of hydrolysed circles of glucomannan and sodium carboxymethylcellulose caused by application of CGTase and its mutants were measured. The morphology of CGTase-treated fungal hyphae was observed with optical or scanning electron microscopy. Each experiment was repeated three times.

4.6 | Visualization and quantification of H2O2, lignification, callose staining, HR, and cell death

To visualize the accumulation of H2O2, leaves from control and treatment plants were collected 48 hr after Vd080 inoculation. After being incubated for 8 hr in DAB (1 mg/ml, pH 7.5), leaves were decolorized at 100 °C in 95% ethanol for 2 min and then 100% ethanol. H2O2 accumulation in leaves was observed under a microscope in 70% glycerol. Each experiment was repeated three times.

Lignification of control and treated plants was examined with phloroglucinol staining. Root sections of cotton seedlings were incubated in 10% phloroglucinol solution for 2 min. The samples were then incubated in concentrated H2SO4 briefly, and the staining was observed using a microscope. Each experiment was repeated three times.
Callose deposition in leaves penetrated by *V. dahliae* was visualized by aniline blue staining (Millet et al., 2010). Leaf disks were immersed in a fixative solution (3:1 ethanol: acetic acid solution) for 2–3 hr. Leaf disks were then rehydrated in 70% ethanol for 2 hr, 50% ethanol for an additional 2 hr, and water overnight. After two or three water washes, leaf disks were treated with 10% NaOH and kept at 37 °C for 1–2 hr to make the tissues transparent. After three or four water washes, leaf disks were incubated for 3–4 hr in 150 mM K$_2$HPO$_4$, pH 9.5, 0.01% aniline blue (Sigma-Aldrich). The leaf disks were mounted on slides, and callose was observed immediately using a UV microscope (excitation 390 nm, emission 460 nm). Each experiment was repeated three times.

Leaves from *A. thaliana* (3 weeks old) (Wang et al., 2012) and *N. benthamiana* were observed for HR (D’Silva & Heath, 1997) and ROS (Thordal Christensen et al., 2010). Each experiment was repeated three times.

Plant cell death was visualized with trypan blue staining (Choi & Hwang, 2011). Leaves were soaked in trypan blue dye (1 g phenol, 1 mg trypan blue, 1 ml lactic acid, 1 ml glycerol dissolved in 1 ml sterile distilled water) and then stained by boiling. After cooling to room temperature, samples were decolorized with a chloral hydrate solution (2.5 g/ml). Each experiment was repeated three times.

## 4.7 | Expression of defence-related genes using RT-qPCR

Leaves were excised 0, 12, 24, 36, 48, 60, and 72 hr after CGTase or protein buffer treatment for RNA extraction. RNA isolation and cDNA synthesis were performed as described previously (Cheng et al., 2017). The relative expression of cotton defence-related genes was analysed using RT-qPCR with specific primers (Table S1) (Cheng et al., 2017; Li et al., 2014a; Zhang et al., 2016). Technical replicates of three independent biological samples were performed.

## 4.8 | *A. thaliana* transformation and molecular analysis

To express CGTase in *A. thaliana*, the CGTase sequence was optimized using an online website (http://www.kazusa.or.jp/codon/) and then synthesized by Sangon Biotech (Shanghai). The 2,154-bp full-length CGTase coding sequence was cloned using the primers OE-CGTase-F and OE-CGTase-R (Table S1). For overexpression studies, the 35S::CGTase vector was constructed by digesting the sequence with XbaI and BamH I (BioLabs). The sequence was then inserted into the pBI121 (Cambia) plant binary vector containing a kanamycin resistance gene with the In-Fusion HD Cloning Plus (Clontech). This vector was transformed into *Agrobacterium tumefaciens GV3101* using the freeze-thaw method. *A. thaliana* Col-0 was transformed with the overexpression vector via the floral dip method (Clough & Bent, 2010). T$_0$–T$_3$ transgenic seeds were then spread evenly on plates of MS medium containing 50 μM kanamycin to select for positive transformants. The false-positive seedlings turned yellow before dying. T$_3$ lines with the transgene and the correct segregation ratio were selected on the basis of CGTase expression assessed with RT-qPCR analysis. Only stable homozygous T$_4$ lines that exhibited high CGTase expression levels were chosen for further functional analysis.

## 4.9 | Plant disease resistance assay

The cotyledons and roots of *G. hirsutum* ‘Jimian 11’ cotton seedlings (2 weeks old) were treated with CGTase injection (20 μl) and root irrigation (10 ml), respectively. Three days later, the cotton seedlings were inoculated with *V. dahliae* Vd080 conidial suspension (2 × 10$^7$ conidia/ml) in a greenhouse. Inoculation with protein buffer was used as a negative control. Roots from at least 30 plants from each treatment group were immersed in spore suspension to assay for *V. dahliae* infection. Each experiment was repeated three times.

* A. *thaliana* plants were inoculated by irrigating roots with spore suspensions adjusted to a concentration of 1 × 10$^7$ conidia/ml. The roots of at least 30 plants from each treatment group were analysed. Each experiment was repeated three times.

DNA of *A. thaliana* was isolated at 28 days postinoculation by the CTAB method. The DNA content of *V. dahliae* was measured by qPCR using *β*-tubulin species-specific primers for *V. dahliae*, and AtUBQ10 as an internal control gene.

Disease severity of seedlings was scored at 28 days postinoculation on the following criteria: 0 = healthy, no symptoms on leaves; 1 = one or two cotyledon leaves showing symptoms; 2 = a single true leaf showing symptoms; 3 = more than two leaves showing symptoms; and 4 = plant death. The overall disease index and control efficacy were calculated as follows:

\[
\text{Disease index} = \left( \frac{(n_0 + n_1 + 2n_2 + 3n_3 + 4n_4)}{4n} \right) \times 100
\]

\[
\text{Efficacy (\%)} = \frac{\text{Disease index}_{\text{control}} - \text{Disease index}_{\text{treatment}}}{\text{Disease index}_{\text{control}}} \times 100
\]

where $n_0$–$n_4$ are the numbers of plants with each of the corresponding disease scores and $n$ is the total number of plants assessed.

## 4.10 | Statistical analysis

Statistical comparisons of quantitative assays for three independent experiments were performed using a mixed-effects model for nested analysis of variance (ANOVA), implemented in R, at a significance level of $\alpha = 0.05$ or 0.01.

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The authors have declared that no competing interests exist.

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.

FIGURE S1 Cluster analysis of CGTase gene and expression of CGTase protein. (a) Phylogenetic analysis of CGTase and its orthologs from other bacteria in NCBI. (b) The content of protein in the fluid after gel filtration. M, maker; 1, sample before gel filtration; 2, sample before molecular sieve

FIGURE S2 Mass spectrogram of concentration of *N*-acetylglucosamine (hydrolysates of chitin) by liquid chromatograph mass spectrometer. Mass spectrogram of concentration of *N*-acetylglucosamine, which from the chitin was treated by domains A1 (a), B (b), A2 (c), C (d), D (e), E (g) mutants, and CGTase (g). (h) The structural diagram of chitin hydrolysed product

FIGURE S3 Screening of stable transgenic T4 lines and the effects of overexpressing CGTase in *Arabidopsis*. (a) Detection of positive lines. (b) The expression levels of CGTase were determined by RT-qPCR using *AtUBQ10* as an internal control gene. The values are the means ± SD, n = 3. The standard deviations were calculated from the results of three independent experiments. Different lowercases indicated significant difference at p < .05 level, t test

TABLE S1 Primers used in this study

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