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A glycoside hydrolase 12 protein from *Cytospora chrysosperma* triggers plant immunity but is not essential to virulence

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Abstract

Phytopathogens secrete numerous effectors that facilitate their infection and colonization processes. However, the pathogenic mechanism of effectors in *Cytospora chrysosperma*, the causal agent of canker disease in many woody plants, remains poorly understood. In this study, we identified five glycoside hydrolase family 12 (GH12) effector genes in *C. chrysosperma* genome, all of which were significantly upregulated during the infection stages. Among them, *CcEG1*, which contains an additional carbohydrate-binding module family 1 domain (CBM1) at the C-terminal, was selected for further analysis. Transient expression studies showed that CcEG1 was localized to the apoplastic region of *Nicotiana benthamiana* and acted as an elicitor to induce cell death, and activate the expression of genes involved in salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) signaling pathways. Furthermore, the GH12 domain (position 43–249) was sufficient for cell death-inducing activity, rather than the CBM1 domain. Additionally, the leucine-rich repeat (LRR) receptor-like kinases *NbBAK1* and *NbSOBIR1* were required for defense responses triggered by CcEG1. Intriguingly, deletion of *CcEG1* did not affect fungal pathogenicity, growth, response to hydrogen peroxide and cell wall integrity agents, but affected cellulase utilization. In conclusion, our results suggest that CcEG1 induces coreceptors *NbBAK1*- and *NbSOBIR1*- dependent plant immunity, increasing our understanding about fungal pathogenesis during the interaction between *C. chrysosperma* and its host.

Keywords *Cytospora chrysosperma*, Glycoside hydrolase 12, Plant immunity, Elicitor

Introduction

Over the course of long-term evolution, plants have developed a mature innate immune system to defense potentially harmful pathogens (Akira et al. 2006; Jones and Dangl 2006; Thomma et al. 2011). The first line of defense involves plant pattern recognition receptors (PRRs) on the cell membrane, which can recognize highly conserved pathogen-associated molecular patterns (PAMPs) or microbe-associated molecular patterns (MAMPs) to trigger a series of downstream immune signals and thereby stimulate PAMPs triggered immunity (PTI) (Kim et al. 2000; Klarzynski et al. 2000; Silipo et al. 2010; Newman et al. 2013). The PTI response is nonspecific and common existent in plant. However, phytopathogens can deliver numerous effectors to suppress the PTI and promote colonization (IpCho et al. 2010; Soyer et al. 2015; Piechocki et al. 2018; Situ et al. 2020; Tintor et al. 2022). To counter this, the nucleotide-binding domain leucine-rich repeat receptors (NLRs) can recognize the

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effectors and trigger the effector triggered immunity (ETI), which is specific and more intense (Yuan et al. 2021a, b). Recently studies have shown that PTI and ETI are mutual related (Tena 2021; Yuan et al. 2021b; Chang et al. 2022). On the other hand, phytopathogens can evolve new effectors or modify or even abandon the previously identified effectors to avoid being recognized.

The plant cell wall serves as an important barrier against phytopathogens, which is comprised of a complex interconnected mixture of proteins and polysaccharides, such as cellulose, hemicellulose, glycosaminoglycans, pectin (Carpita 2011; Burton and Fincher 2012). However, many filamentous fungi have developed the ability to degrade plant cell wall material by secreting large number of cell wall-degrading enzymes (CWDEs) to facilitate infection. These enzymes can be classified into four types: cutinase, pectinase, cellulase, hemicellulose, according to the different substrates (Lynd et al. 2002; Passos et al. 2009). Cutinase is a serine esterase that degrades fatty esters and triglycerides, which is often associated with the pathogenicity of plant pathogenic fungi (Xin et al. 2021). For example, overexpression the cutinase gene *MfCUT1* could increase the virulence of the brown rot pathogen *Monilinia fructicola* on *Prunus* spp. (Lee et al. 2010). Pectinase is a compound enzyme and can be divided into two categories, one is pectin esterase and polygalacturonase that existing in higher plants and microorganisms, the other is pectin lyase that existing in microorganisms (Swain et al. 2009; Huang et al. 2019). Pectinases secreted by fungi and bacteria can cause plant rot and cell necrosis (Wattad 1995; Reignault et al. 2000; Joshi et al. 2015). In *Colletotrichum magna*, deletion of the pectin lyase gene resulted in significant reduction in fungal virulence (Wattad 1995). Additionally, both pectate lyase VdPEL1 and cutinase VdCUT11 from *Verticillium dahliae* contributes to fungal virulence and simultaneously activated plant immunity as PAMPs (Muchero et al. 2018; Yang et al. 2018). Hemicellulase is a general term for a class of enzymes that degrades hemicelluloses into xyloglucan, xylan, and galactomannan, mainly including xylanase and mannanase (Gostinčar et al. 2014; Pavarina et al. 2021). Xylanase Xyn11A contributes to the infection of *Botrytis cinerea* through a conserved 30-amino acids region on the enzyme surface, and its ability to induce cell death is independent of hydrolytic activity (Noda et al. 2010). Cellulases are a group of complex enzymes such as the endoglucanase, exoglycanase, β-1,4-glucosidase and auxiliary enzymes (Sharma et al. 2016; Dumorné et al. 2017). Among them, glycoside hydrolase (GH) family is one the most important type with numerous members, including GH3, GH6, GH12, GH61, many previous studies had shown that the glycoside hydrolases are required for fungal virulence (Minic and Jouanin 2006; Van Vu et al. 2012; Zhang et al. 2021; Bradley et al. 2022).

Previous studies indicate that GH12 proteins are widely distributed in microorganism, many of these GH12 proteins could induce cell death in *Nicotiana benthamiana* (Ma et al. 2015). In *Phytophthora sojae*, the GH12 effector XEG1 was found to trigger plant defense responses and induce cell death through a *BAK1*-dependent manner (Ma et al. 2015). Meanwhile, VdEG1 and VdEG3 belonging to GH12 family could also be recognized by receptor-like kinases *BAK1* and *SOBIR1* in *V. dahliae*, respectively (Gui et al. 2017). Additionally, *BAK1* and *SOBIR1* were both required for FoEG1 (a GH12 effector from *Fusarium oxysporum*) induced cell death in *N. benthamiana* (Zhang et al. 2021). Many cellulases are composed of catalytic domain and substrate binding domain like carbohydrate-binding module (CBM). The function of CBM is binding with the substrate more tightly, and then the catalytic domain can active the enzyme to initiate the degradation process (Sandgren et al. 2003; de Almeida et al. 2013). Besides, the carbohydrate-binding module family 1 (CBM1) protein domain in *V. dahliae* was proved to suppress GH12 protein-induced cell death (Gui et al. 2017). In *N. benthamiana*, the LRR receptor-like protein RXEG1 was identified to specifically recognize the glycoside hydrolase 12 protein XEG1 via the LRR domain in the apoplast. They could form a complex with the LRR receptor-like kinases *BAK1* and *SOBIR1* to transduce the XEG1-induced defense signal (Wang et al. 2018a). These studies indicate that the glycoside hydrolase 12 family proteins are involved in the infection of phytopathogens and activation of plant immunity.

Cytospora chrysosperma is a necrotrophic pathogenic fungus that causes stem canker disease in over 80 tree species worldwide (Adams et al. 2006; Kepley et al. 2015; Wang et al. 2015). Generally, *C. chrysosperma* infects host plants through wound, leading to the formation of serious ring spots and watermarks. In the late stages of disease cycle, the fungus generates many conidia on the branches of the hosts and spread through wind and rain (Madar et al. 2004; Tian et al. 2014). Our previous studies have shown that the MAPK-Pmk1 signal pathway is essential for the virulence of *C. chrysosperma*, which can regulate the downstream virulence factors such as transcription factors, effectors, and secondary metabolism related components (Yu et al. 2019; Xiong et al. 2021; Yu et al. 2022). However, the molecular mechanism involved *C. chrysosperma*-host interaction remains unclear.

This study presents the functional characterization of CcEG1, a secreted GH12 family protein from *C. chrysosperma* that could trigger plant cell death with its GH12 domain alone. Additionally, we found that the elicitor

activity of CcEG1 requires its apoplastic localization and the presence of PRRs *NbBAK1* or *NbSOBIR1*. These results provide insights into the molecular mechanisms of *C. chrysosperma*-host plant interaction and highlight the importance of CcEG1 in the pathogenicity of *C. chrysosperma*.

Results

The GH12 family genes were significantly up-regulated during the infection stages of *C. chrysosperma*

Through homologous blast analysis, five genes containing GH12 domain were identified in the genome of *C. chrysosperma* (NCBI accession number: JAEQMF010000000). All these five genes (*CCG_9229*, *CCG_3099*, *CCG_3939*, *CCG_6517*, *CCG_2250*, named *CcEG1–5*) contained a GH12 domain and a signal peptide but lacking transmembrane domain. Remarkably, CcEG1 possesses six cystine residues and a CBM1 domain at C-terminal (Fig. 1a). In order to determine the putative functions of these five genes during the infection process, their relative expression levels were analyzed. The results showed that all the five genes were significantly up-regulated at the early stages of infection (Fig. 1b), suggesting that these five GH12 members are involved in the infection processes of *C. chrysosperma*. Among them, only *CcEG1* contains a CBM1 domain at C-terminal, therefore it was chosen for further analysis.

CcEG1 targeted the apoplast of *N. benthamiana* to induce cell death

In this study, we focused on the CcEG1 that containing a CBM1 domain at the C-terminal. To explore the function of GH12 family candidate effector CcEG1, we examined the secretion ability of its signal peptide by yeast signal trap assay. As shown in Fig. 2a, the yeast YTK12 containing recombinant pSUC2 with the signal peptide of CcEG1 could grow on the YPRAA medium as the positive control Avr1b, demonstrating that the signal peptide of CcEG1 was functional and CcEG1 could be secreted into the extracellular space (Fig. 2a). Then, we deleted the N-terminal signal peptide of CcEG1 to produce CcEG1$^{\Delta SP}$ or replaced it with the signal peptide from plant pathogenesis-related protein 1 (PR1) to produce PR1^{SP}-CcEG1$^{\Delta SP}$ (Fig. 2b). All the fragments were cloned into pGR106 and transformed into the *Agrobacterium tumefaciens* GV3101, and then agroinfiltrated into the *N. benthamiana* leaves. The results demonstrated that the full length of CcEG1 and PR1^{SP}-CcEG1$^{\Delta SP}$ specifically triggered cell death in *N. benthamiana* at 3 days post inoculation (dpi), whereas CcEG1$^{\Delta SP}$ did not induce cell death as the negative control (Fig. 2c). Western blot confirmed that all proteins were expressed in *N. benthamiana* (Fig. 2d). Subsequently, we investigated the subcellular localization of CcEG1 in *N. benthamiana* by fusion the CcEG1 with a C-terminal GFP. The CcEG1$^{\Delta SP}$-GFP4, CcEG1-GFP4 and PR1^{SP}-CcEG1$^{\Delta SP}$-GFP4 were transiently expressed in *N. benthamiana* leaves with the empty pBINGFP4 as a negative control. The leaves were treated by 1 M NaCl or water for five minutes before observation, respectively. Confocal microscopy results showed that CcEG1$^{\Delta SP}$-GFP4 was localized in the intracellular region with NaCl or water treatment, while CcEG1-GFP4 and PR1^{SP}-CcEG1$^{\Delta SP}$-GFP4 were localized in the apoplastic region after plasmolysis (Additional file 1: Figure S1). Collectively, the results indicated that CcEG1 targets to the apoplast of *N. benthamiana* to induce cell death.

Transient expression of CcEG1 could induce plant immunity responses

Hypersensitive reaction (HR) is a type of programmed cell death and an early plant defense response that often

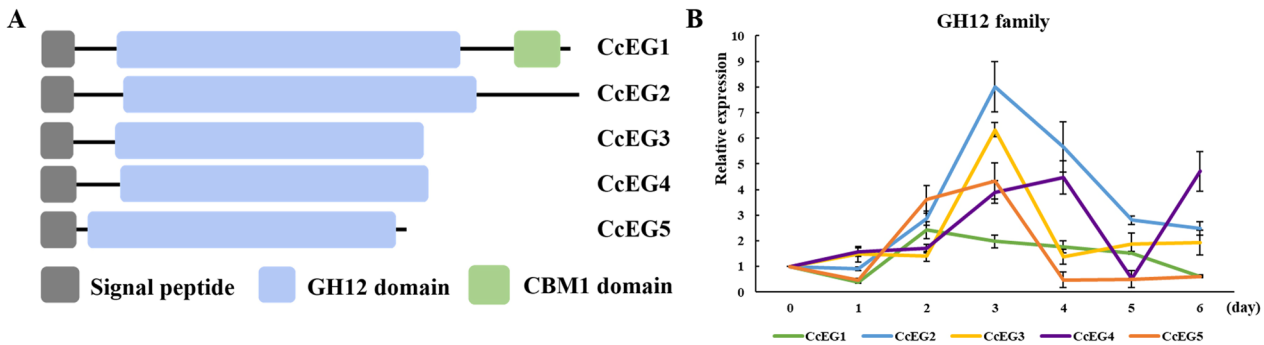

Fig. 1 Five GH12 family candidate effectors were identified and up-regulated during infection. **a** Schematic diagram of the five GH12 family candidate effectors, analysis by Interpro database. **b** The relative expression level of GH12 family candidate effectors during the infection process. The expression level was detected by reverse transcription-quantitative polymerase chain reaction (RT-qPCR). RNA was extracted after 0, 1, 2, 3, 4, 5, 6 days of inoculation of poplar branches with plugs of *C. chrysosperma*

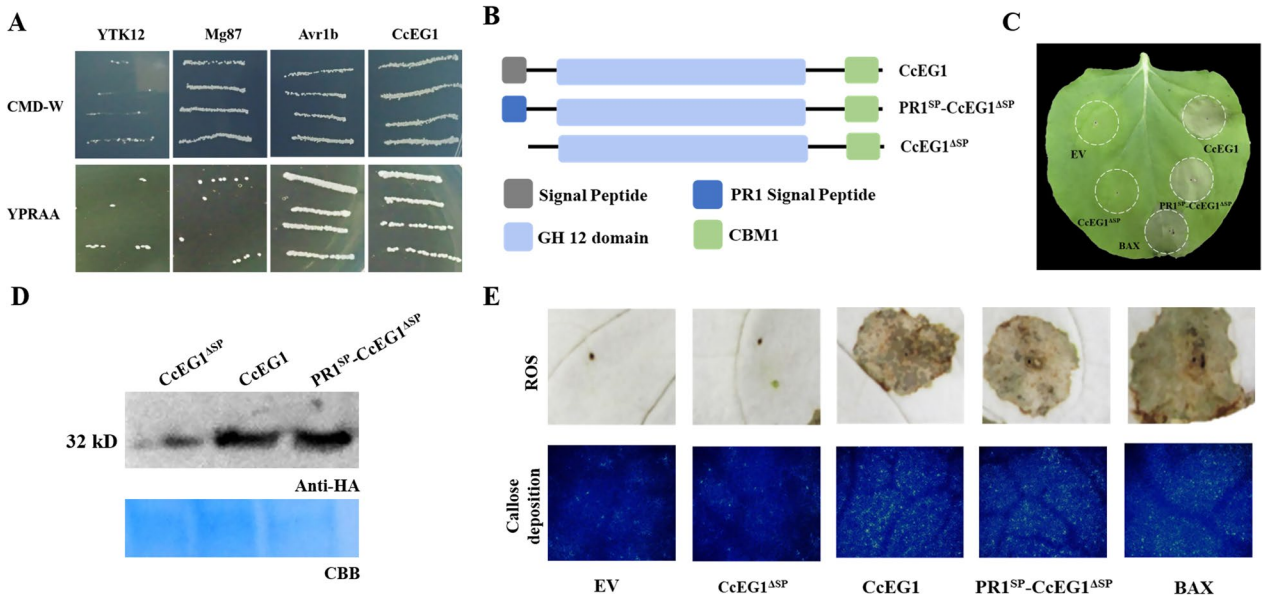

Fig. 2 CcEG1 induce plant defense response in *N. benthamiana* with signal peptide. **a** Verification of secretion activity of CcEG1 signal peptide by yeast signal trap assay. Yeast strain YTK12 cannot grow on medium using sucrose as the only carbon source, but when plasmid Psuc2 that lacking the signal peptide and the initiation codon ATG could ligate to a functional signal peptide the and then introduced into YTK12, the yeast strain can grow normal. The known functional SP of Avr1b was used as a positive control, Mg87 was set as negative control. **b** The cell death-inducing ability of CcEG1 was determined in *N. benthamiana*. BAX and pGR106 (EV) were chosen as positive and negative controls, respectively. Photographs were taken 5 days post agroinfiltration. **c** Western blot of proteins from *N. benthamiana* leaves transiently expressing CcEG1, CcEG1$^{\Delta SP}$, PR1^{SP}-CcEG1$^{\Delta SP}$ fused with 3*HA tags. **d** Schematic diagram of CcEG1 with or without signal peptide, and replacing by pathogenesis-related protein 1 (PR1). **e** CcEG1 can induce plant immune defense response when it contains functional signal peptide. BAX and pGR106 (EV) were chosen as positive and negative controls, respectively. The picture was photographed 24 h after the infiltration. Data represented have three independent biological repeats

accompanied by, ROS burst and callose deposition (Bousette et al. 2010; Mayer et al. 2018). To further investigate whether CcEG1-induced cell death was associated with plant defense responses, the ROS accumulation and callose deposition were measured 24 h after infiltration CcEG1 in *N. benthamiana* (Fig. 2e). The results showed that both CcEG1 and PR1^{SP}-CcEG1$^{\Delta SP}$ could induce ROS accumulation and callose deposition, suggesting that CcEG1-induced cell death is related to the plant defense responses.

Subsequently, we examined the expression of the marker genes *NbHIN1* and *NbHSR203J*, which are involved in HR. The results demonstrated that they were highly up-regulated after infiltration of CcEG1, which revealed that CcEG1 can induce HR-related immunity responses in *N. benthamiana* (Fig. 3). Previous works have shown that several GH12 members were identified as PAMP, therefore, we also calculated the expression of marker genes involved in PTI response after infiltration of CcEG1. The results showed that *Nbpti5, NbWRKY7, NbWRKY8*, and *NbAcre31* were all highly expressed in *N. benthamiana* after transient expression of CcEG1 (Fig. 3). Additionally, we found that *NbPR1*, *NbPR2*, *NbPR4*, *NbLOX*, and *NbERF1*, the marker gene of salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) signal pathway, were also significantly up-regulated in *N. benthamiana* after transient expression of CcEG1 (Fig. 3). These results indicating that CcEG1 acts as an elicitor to induce plant PTI response and then transmits immunity signals through SA, JA and ET signaling pathway.

CcEG1 was not associated with pathogenicity of *C. chrysosperma*

To investigate the potential functions of CcEG1, the mutant strains of *CcEG1* were constructed by replacing the target gene with hygromycin cassette through split-marker method (Fig. 4a). Two successful *CcEG1* deletion mutants Δ*CcEG1*_14 and Δ*CcEG1*_19, were generated and confirmed by Southern blot analysis and PCR amplification with internal and external primer pairs (Fig. 4b, c). The complementary strain Δ*CcEG1*/C was also obtained by split-marker method using Δ*CcEG1*_19 mutant as the recipient strain.

To elucidate whether *CcEG1* contributed to the pathogenicity of *C. chrysosperma*, mycelial plugs of WT, Δ*CcEG1*_14, Δ*CcEG1*_19, and Δ*CcEG1*/C strains were inoculated on the branches and leaves of *Populus tomentosa* (Fig. 4d, f). However, no obvious differences were

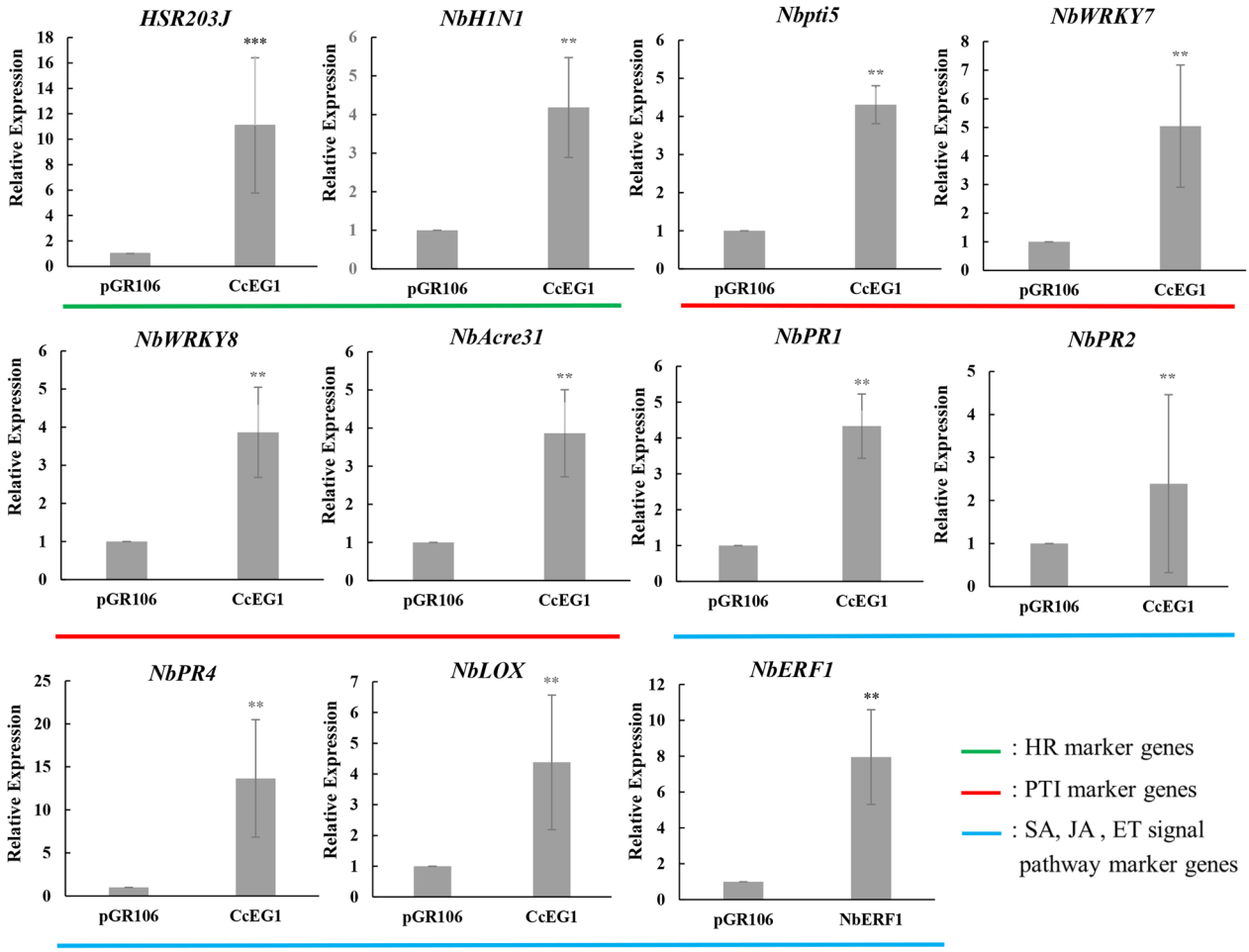

Fig. 3 CcEG1 acts as an elicitor to induce plant immune responses. **a** Relative expression of HR marker genes in *N. benthamiana*. **b** Relative expression of PTI response marker genes in *N. benthamiana*. **c** Relative expression of JA, SA and ET signal pathway marker genes in *N. benthamiana*. The totally RNA of *N. benthamiana* leaves were extracted 24 h after inoculation. $^{**}P < 0.01$; $^{***}P < 0.001$

observed among the WT, *CcEG1* deletion mutants and complementary strains (Fig. 4e, g). Furthermore, we collected the GH12 members which had been functional characterized in other phytopathogens. As shown in Table 1, five out of seven GH12 proteins were required for virulence. but CcEG1 and BcXYG1 were not. To examine whether the difference in virulence might result from the sequence polymorphism, we performed multiple sequences alignment of the GH12 domain of these proteins. However, no dramatical sequence differences and two conserved enzyme active sites were observed. Additionally, we found that the *N*-glycosylation of the GH12 proteins was not conserved among these members which prevented the protein from being degraded in the apoplastic regions (Fig. 4h). Intriguingly, a predicted conserved phosphorylation site PxS/T was found among them, but it was not the case in Ps_109281 which could induce plant cell death (Wang et al. 2022). These results indicated that CcEG1 is not a key virulence-related factor in *C. chrysosperma*.

CcEG1 did not affect the growth and stress response of *C. chrysosperma*, but was required for the utilization of cellulose

To determine the functions of CcEG1 in fungal growth and stress responses, mycelial plugs of WT, Δ*CcEG1_14*, Δ*CcEG1_19*, and Δ*CcEG1/C* were inoculated on PDA medium at 25°C for three days. The statistical data showed that no significant difference was observed in mycelial growth among these strains, indicating that CcEG1 is not required for the growth of *C. chrysosperma* (Additional file 1: Figure S2a). Subsequently, we assessed the response of each strain to the hydrogen peroxide (H_2O_2), and cell wall integrity inhibited agents calcofluor white (CFW) and Congo red (CR). The Δ*CcEG1* mutants exhibited no distinguish differences in responses to the

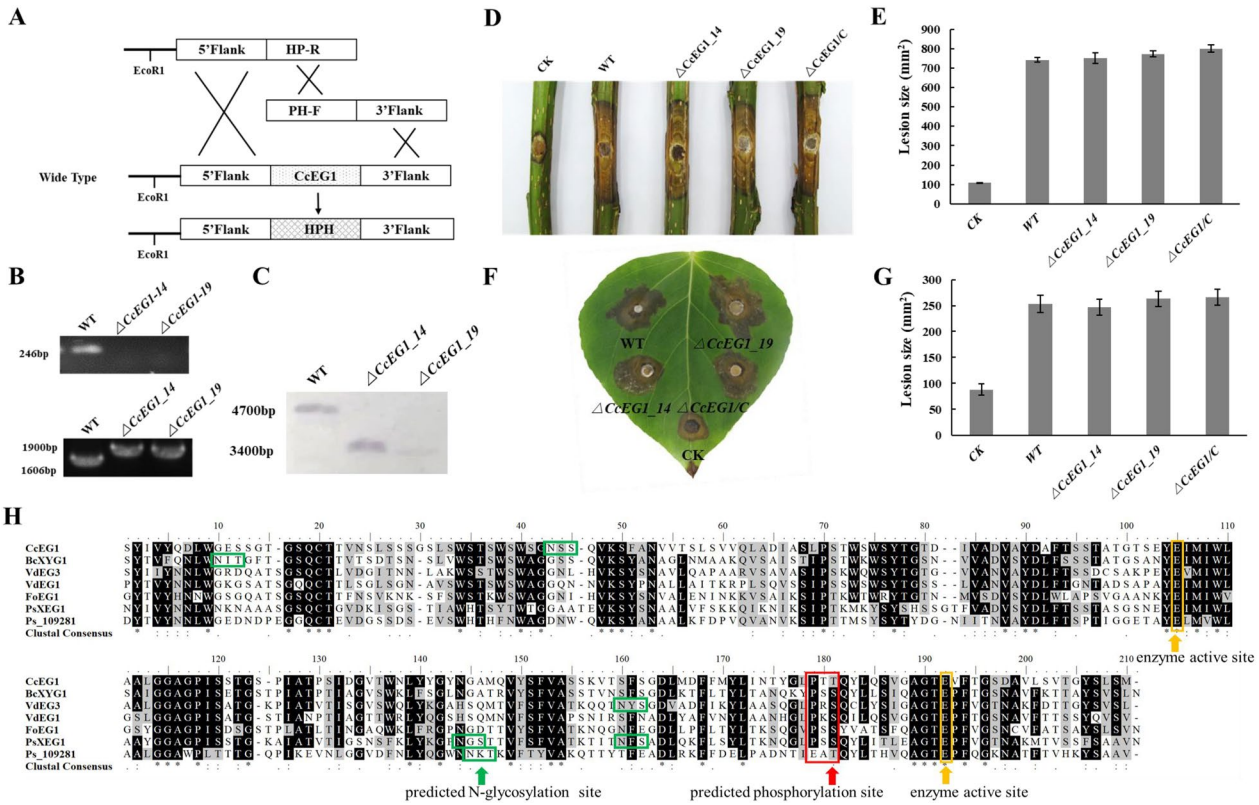

Fig. 4 CcEG1 has no effect on the pathogenicity of *C. chrysosperma*. **a** CcEG1 mutant was constructed by the method of Split marker to replace target gene with hygromycin cassette based on the principle of homologous recombination. **b**, **c** CcEG1 single-copy mutants were verified by internal and external primers and Southern blot. **d**, **f** Inoculated poplar branches or leaves with plugs of wild-type, CcEG1 mutants, and complemented strains, agar was set as negative control. The lesion areas were measured at 5 dpi. **e**, **g** Quantification of colony diameter in poplar twigs or leaves that inoculated with WT, CcEG1 mutants, and complemented strains. This experiment was performed three times with similar results, each assay was performed on at least three independent biological repeats. The statistical analyses were conducted by SPSS v16.0, and Duncan s test at $P \leq 0.05$ or $P \leq 0.01$ was used for determining the differences between mutants and WT strain. **h** Multiple sequence alignment of GH12 domain from functional characterized GH12 members

Table 1 Functional characterized GH12 members in different phytopathogens

Gene ID	Species	Induce cell death	Virulence of deletion mutant	Virulence of overexpression strain	References
CcEG1	*C. chrysosperma*	Yes	Not changed	Not application	This study
BcXYG1	*B. cinerea*	Yes	Not changed	Not changed	Zhu et al. (2017)
VdEG1	*V. dahliae*	Yes	Reduced in cotton Enhanced in *Nicotiana benthamiana*	Not application	Gui et al. (2017)
VdEG3	*V. dahliae*	Yes	Reduced in cotton Enhanced in *Nicotiana benthamiana*	Not application	Gui et al. (2017)
FoEG1	*F. oxysporum*	Yes	Reduced	Not application	Zhang et al. (2021)
PsXEG1	*P. sojae*	Yes	Reduced	Reduced	Ma et al. (2015, 2017)
Ps109281	*P. sojae*	No	Reduced	Not application	Wang et al. (2022)

H_2O_2, CFW and CR compared with WT and complementary strains, indicating that CcEG1 is not involved in fungal growth and stress responses in *C. chrysosperma* (Additional file 1: Figure S2b–d).

Previous studies had shown that the GH12 family proteins displayed conserved glycoside hydrolase, which depends on the two conserved enzyme active sites. In this study, we determined the enzymatic activity of CcEG1

through plate assays. The 3-mm mycelium plugs of WT, Δ*CcEG1*_14, Δ*CcEG1*_19, and Δ*CcEG1*/C strains were cultured in minimal methanol medium (MM medium) supplemented with glucose, CMC-Na and cellulose as the only carbon source at 25°C for 5 days, separately. As shown in Fig. 5, comparable colony diameters were observed among each strain when grown on the MM supplemented with glucose. However, the Δ*CcEG1*_14 and Δ*CcEG1*_19 showed obvious defects in fungal growth compared with WT and complementary strains when grown on the MM supplemented with CMC-Na and cellulose, indicating that deletion of *CcEG1* would compromise the utilization of cellulose. The results suggest that CcEG1 may also possess the glycoside hydrolase activity.

GH12 domain of CcEG1 was sufficient for its elicitor function in *N. benthamiana*

Pattern-recognition receptors (PRRs) often recognize specific small epitopes of PAMPs (Orosa et al. 2018; Palmer et al. 2019; Fite et al. 2021). To determine whether the full-length of CcEG1 was needed to generate cell death-inducing activity, the GH12 domain and CBM1 domain were truncated, respectively. Then we generated the CcEG1-Δcbm1, CBM1 and SP-CBM1 containing *A. tumefaciens* strains, and infiltrated into *N. benthamiana* leaves (Fig. 6a). The results demonstrated that CcEG1 lacking the CBM1 domain (CcEG1-Δcbm1) could still trigger cell death, while both the CBM1 and SP-CBM1 were failed to induce cell death (Fig. 6b). ROS burst and callose deposition were also detected and proved to be consistent with the cell death-inducing activity (Fig. 6c). Western blot confirmed that all proteins were expressed in *N. benthamiana* (Fig. 6d). Subsequently, the relative expression of HR and PTI marker genes were examined between the negative control (pGR106 empty vector) and SP-CBM1, no significant difference was observed among the expression levels of these genes, indicating that they were not induced when transient expression the SP-CBM1 (Additional file 1: Figure S3). These results suggesting that the GH12 domain was sufficient for CcEG1 to induce cell death in *N. benthamiana*, while the CBM1 domain was not essential for CcEG1 to activate host immunity.

NbBAK1 or NbSOBIR1 of *N. benthamiana* were required for CcEG1 induced cell death

Previous works proved that GH12 members could form a complex with the LRR receptor-like kinases BAK1 and SOBIR1 to transmit the PAMP-induced defense signal (Liebrand et al. 2014; Wang et al. 2018a). To confirm that CcEG1 functioned as a putative PAMP to participate in the induction of plant cell death. Virus-induced gene silencing (VIGS) of *NbBAK1* or *NbSOBIR1* was performed in *N. benthamiana* using a tobacco rattle virus (TRV) vector. Then the CcEG1 was transiently expressed

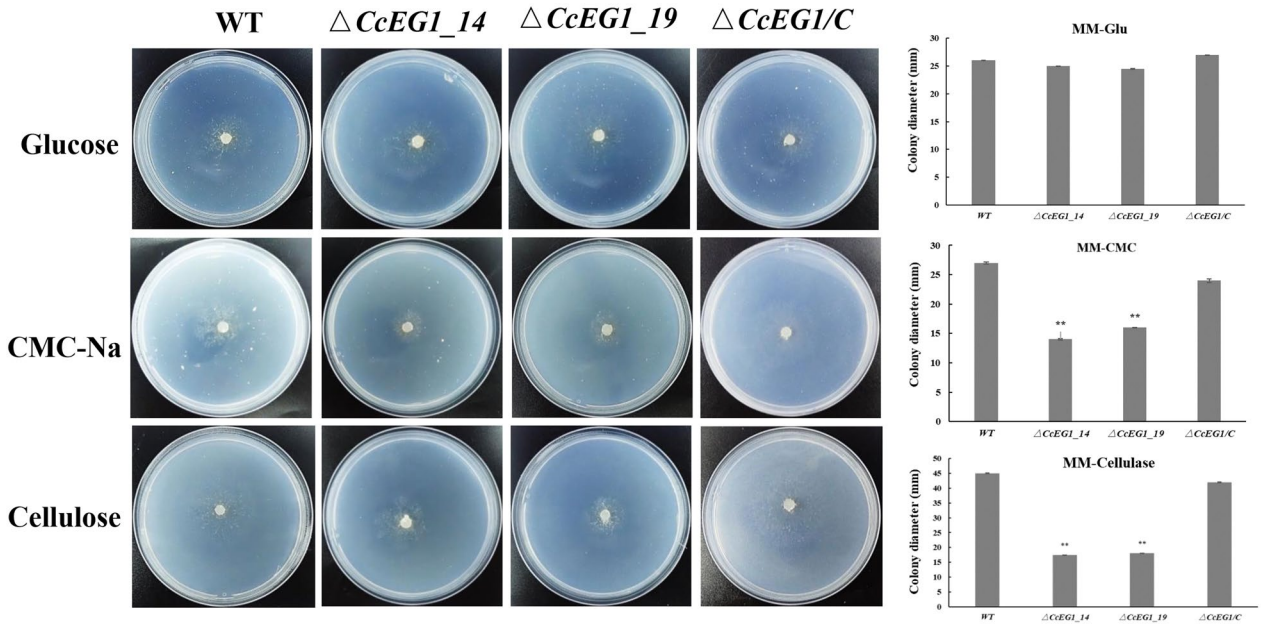

Fig. 5 CcEG1 can degrade cellulose and carboxymethyl cellulose as carbon source. 3-mm-diameter *C. chrysosperma* mycelial plugs was placed on the center of minimal methanol medium (MM) with glucose, CMC-Na or cellulose as the sole carbon source. This experiment was performed three times with similar results, and each assay was performed on at least three independent biological repeats. $^{**}P < 0.01$

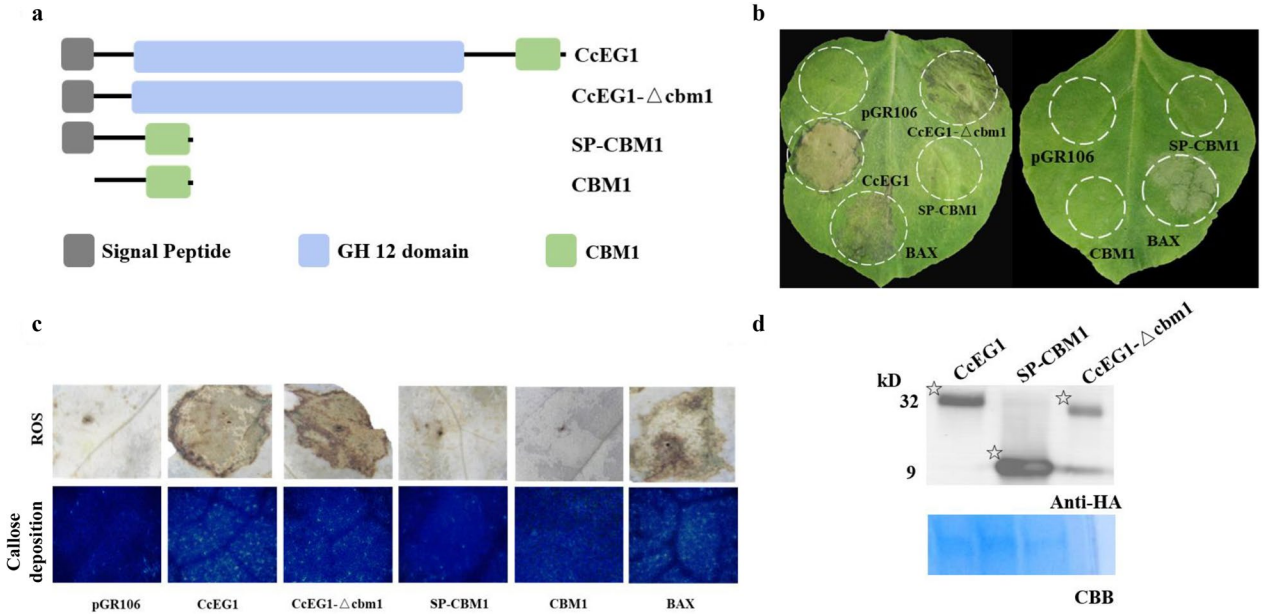

Fig. 6 The GH12 domain was sufficient for CcEG1 to induce cell death in *N. benthamiana*. **a** Schematic diagram of CcEG1 mutant. **b** Cell death-inducing activity of the CcEG1 mutant were observed 5 days after agroinfiltration in *N. benthamiana*, *BAX*, and *pGR106* were used as positive and negative controls, respectively. **c** The detection of ROS and callose deposition in *N. benthamiana* leaves at 24 h post inoculation. **d** Western blot of proteins from *N. benthamiana* leaves transiently expressing CcEG1, CcEG1-Δcbm1 and SP-CBM1 with 3*HA tags

on the *NbBAK1* or *NbSOBIR1* silencing plants. As shown in Fig. 7, CcEG1 failed to induce cell death in the *NbBAK1* or *NbSOBIR1* silencing *N. benthamiana*. The expression of *BAK1* and *SOBIR1* was confirmed to be significantly reduced compared with *TRV2: GFP* according to RT-qPCR analysis (Fig. 7b, e). Western blot confirmed that all proteins were expressed in *N. benthamiana* (Fig. 7c, f). These results demonstrated that NbBAK1 and NbSOBIR1 were required for CcEG1-induced cell death in *N. benthamiana.*

Discussion

C. chrysosperma is a necrotrophic pathogenic fungus that usually invades into the host plants through the wounds (Kepley et al. 2015; Han et al. 2021; Xu et al. 2022). Genome data analysis has shown that *C. chrysosperma* encodes multiple CWDEs, including glycoside hydrolases, glycosyl transferases, and polysaccharide lyases. Previous studies have proved that GH12 family candidate effectors have conserved cell wall degradation activity in fungi, which usually contribute to the infection and colonization of phytopathogens (Gui et al. 2017; Yang et al. 2017). However, the function of GH12 family members in *C. chrysosperma* remains unclear. In this study, we investigated the function of GH12 family effector during the interaction of *C. chrysosperma* and plants. We identified five GH12 family candidate effectors in *C. chrysosperma*. Among them, CcEG1 containing a GH12 domain and a CBM1 domain, was acted as an elicitor which was targeted in the apoplast of plants to induce cell death and stimulated downstream immune responses in *N. benthamiana*.

Pathogens utilize effector proteins as molecular weapons to manipulate the host immune responses. The necrotrophic pathogenic fungi usually obtain the nutrients from the dead plant cells. For example, *B. cinerea* reduced plant resistance by activating EDS1- and SGT1-mediated cell death immune signaling pathways to facilitate its infection (El Oirdi and Bouarab 2007). In *C. chrysosperma*, we found that the candidate effectors CcCAP1 and GH61 family effector CcSp84 could regulate the plant immunity therefore to reduce or enhance the plant defense responses (Han et al. 2021; Xu et al. 2022). In this study, we found CcEG1, a cell death inducer that was significantly up-regulated during the early stages of infection, indicating its potential roles in pathogenicity of *C. chrysosperma*.

In plant-pathogen interactions, the apoplastic space represents a complex battlefield where substantial interaction occurs, including the interaction between hydrolytic enzymes and plants receptors (Doehlemann and Hemetsberger 2013; Ma et al. 2017). Soybean produces GmGIP1, an apoplastic glucanase inhibitor protein, which binds to the GH12 family effector PsXEG1 to moderate the virulence of *P. sojae*. However, *P. sojae* would secrete a paralogous PsXEG1-like protein, PsXLP1, which

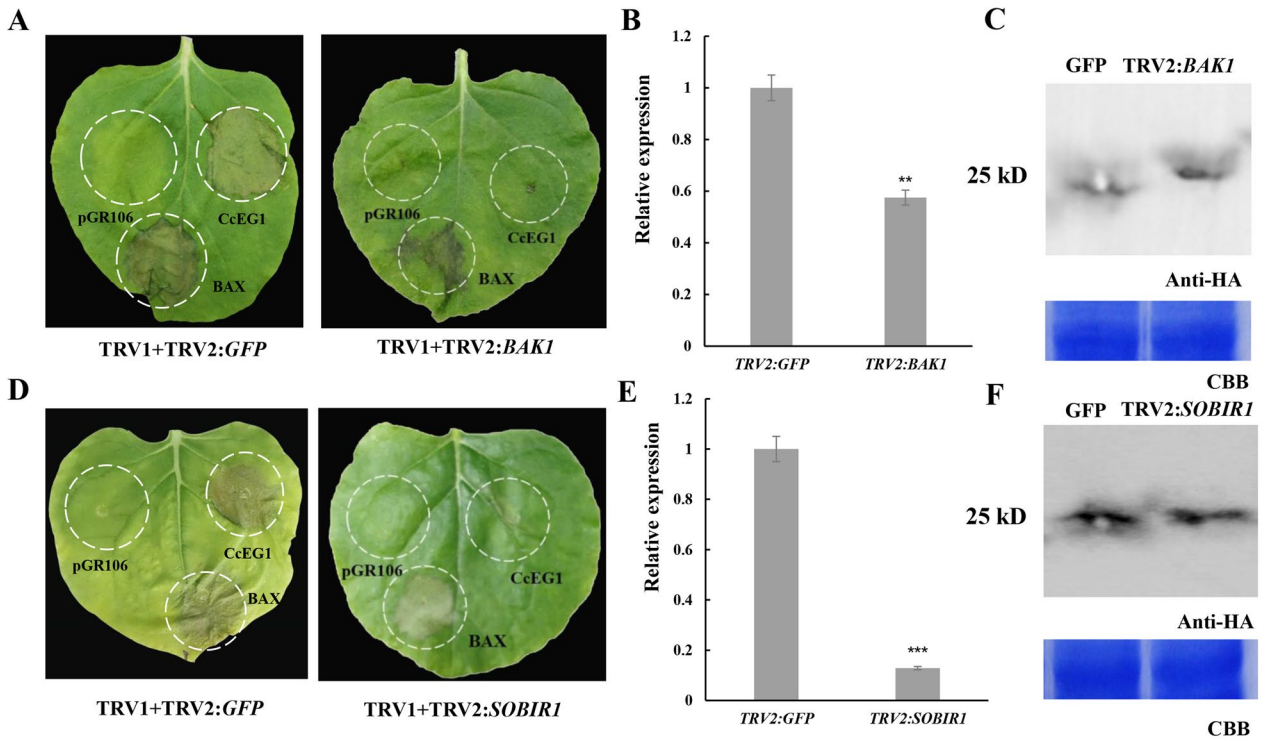

Fig. 7 CcEG1 requires the receptor-like kinases *BAK1* and *SOBIR1* to induce plant immune responses in *N. benthamiana*. **a**, **d** After three weeks silencing *BAK1* and *SOBIR1* by virus-induced gene silencing (VIGS), CcEG1 was inoculated in *N. benthamiana* leaves, and the results showed that it could not induce cell death. **b**, **e** The silencing efficiencies of *BAK1* and *SOBIR1* were determined by quantitative RT-qPCR. Three biological and statistical replicates were performed. **c**, **f** Western blot of CcEG1 protein fused with 3*HA tag that transiently expressed in *BAK1* or *SOBIR1* silenced *N. benthamiana* leaves. The total protein is shown by Coomassie brilliant blue (CBB). $^{**}P < 0.01$, $^{***}P < 0.001$

lacking the enzyme activity but could bind to GmGIP1 more tightly than PsXEG1, resulting in the release of PsXEG1 to support *P. sojae* infection (Ma et al. 2015, 2017). The PsXEG1-PsXEG1-like protein model was conserved in *Phytophthora* species but has not been found in fungi. Another glycoside hydrolase family 12 protein FoEG1 found in *F. oxysporum* was acted as a PAMP that targeted to the apoplast of plants to induce cell death, and contributed to the virulence of *F. oxysporum* through its enzyme activity (Zhang et al. 2021). The GH12 family protein CcEG1 from *C. chrysosperma* was also proved to induce cell death in apoplast of host plants and the host immune response. Similar results were also found in several GH12 family proteins in other phytopathogens, indicating more GH12 proteins possessing elicitor activity in phytopathogens.

Previous studies have proved that GH12 family genes are conserved in phytopathogens. In this study, we collected 58 GH12 family genes from 12 fungal species and 2 oomycetes (Additional file 2: Table S1). As shown in Additional file 1: Figure S4a, 9 out 12 fungi contained 3–6 GH12 genes, while *Candida albicans* and *Saccharomyces cerevisiae* did not possess any GH12 genes. Importantly, there were 12 GH12 genes in *Phytophthora sojae* but only 6 GH12 genes were identified in *Phytophthora infestans*. Among the 58 GH12 genes, only five genes carried a CBM1 domain in addition to the GH12 domain, including *CcEG1* from *C. chrysosperma*, VDAG_07406 from *V. dahliae*, MGG_10972 from *Magnaporthe oryzae*, VP1G_03473 from *Valsa pyri*, and VM1G_09037 from *Valsa mali* (Additional file 1: Figure S4a). Phylogenetic analysis revealed that the GH12 genes from fungi and oomycetes were clearly separated into two clades (Additional file 1: Figure S4b). And 9 members could induce plant cell death according to the previous studies including 5 oomycete GH12 members and 4 fungal GH12 members (Ma et al. 2015; Gui et al. 2017). Remarkably, both of the reported GH12 members (VDAG_07406 and MGG_10972) that containing the CBM1 domain could induce plant cell death (Additional file 1: Figure S4a). These results indicate that the GH12 family genes are widespread in plant pathogenic fungi and oomycetes, but only few members containing a CBM1 domain at C-terminal coupling with GH12 domain. However, the functions of the CBM1 domain in a GH12 protein need to be further analyzed. In *V. dahliae*, the CBM1 domain

contained in the GH12 protein VdEG3 was proved to suppress VdEG1 and VdEG3-induced cell death (Gui et al. 2017). In *C. chrysosperma*, the deletion of CBM1 domain in CcEG1 did not alter the ability to induce cell death, and the GH12 domain was sufficient to perform this function, while only the CBM1 domain was incapable of triggering plant immune response.

PRRs are the plant-derived cell-surface pattern recognition receptors, including receptor-like kinases (RLKs) and receptor-like proteins (RLPs) (Wang et al. 2018b; Lu et al. 2020). BAK1 is a well-known co-receptor for ligand-binding RLKs, and has been found to be recruited to activated RLP/SOBIR1 complexes, which have been proposed to function as two-component RLKs (Chinchilla et al. 2009; Albert et al. 2015; Postma et al. 2016; Wang et al. 2018a). GH12 family effectors displayed partially conserved biological functions during the interaction between pathogen and host, including the ability to induce cell death and recognized by common host receptor like protein or kinases. In *F. oxysporum*, the cell death inducing ability of GH12 family effector FoEG1 was regulated by leucine-rich repeat (LRR) receptor-like kinases BAK1 and SOBIR1(Zhang et al. 2021). In *P. sojae,* XEG1 could induce plant defense responses in a BAK-dependent manner, and Ps109281 could be also recognized by the membrane localized receptor like protein RXEG1 (Ma et al. 2017; Wang et al. 2022). Therefore, SOBIR1 and BAK1 are involved in the regulation of GH12 members induced cell death. In this study, our results proved that NbBAK1 or NbSOBIR1 was essential for the CcEG1 inducedcell death in *N. benthamiana*. Silencing *BAK1* or *SOBIR1* prevented CcEG1 from inducing cell death.

The novel and non-conservative function of effectors from GH12 family demonstrates the diversity of the GH12 family and may have been evolving during the interaction between pathogen and host. Generally, the hydrolytic activity of GH12 family is common, but the relationship between the hydrolase activity and pathogenicity of pathogens is still uncertain. For example, the GH12 family effector XEG1 and FoEG1 from *P. sojae* and *F. oxysporum* were identified to induce cell death, while Ps109281 of *P. sojae* was unable to induce cell death. The hydrolytic activity of VdEG1 from *V. dahliae* was unrelated to the virulence of *V. dahliae*, but the hydrolytic activity of FoEG1, PsEXG1 and Ps109281 were proved to be highly correlated with their virulence, separately (Ma et al. 2015; Zhang et al. 2017, 2021; Wang et al. 2022). In our study, we demonstrated that CcEG1 had the glucoside hydrolase activity, while CcEG1 was not required for virulence of *C. chrysosperma*. The above works reported the various and different functions of GH12 family effectors. Similar results were also found in the BcXYG1 from *B. cinerea* (Zhu et al. 2017). *C. chrysosperma* and *B. cinerea* were necrotrophic pathogenic fungi, while *V. dahliae*, *P. sojae*, and *F. oxysporum* were hemibiotrophic pathogenic fungi. Additionally, different virulence of the same GH12 deletion mutants were observed. The VdEG1/VdEG3 deletion mutants showed increased fungal virulence on *N. benthamiana*, but showed reduced fungal virulence on cotton (Gui et al. 2017). Therefore, we speculated that the differential roles of GH12 proteins in virulence in different phytopathogens might result from the various biological characteristics of each strain and host plant.

The plant immunity regulation activity of effectors may not relate to their pathogenicity in the phytopathogens. For example, the expression of VdSCP27 and VdSCP126 from *V. dahliae* in *N. benthamiana* could result in cell death accompanied by a reactive oxygen species burst, callose deposition, and induction of defense genes, but the signal deletion of VdSCP27 or VdSCP126 failed to affect the fungal virulence on *N. benthamiana* or *Gossypium hirsutum* (Wang et al. 2020). VmHEP1 from *V. mali* was found as a cell death inducer, VmHEP1 deletion mutants did not lead to virulence reduction, but the double deletion of VmHEP1 and VmHEP2 notably attenuated *V. mali* virulence on both apple twigs and leaves (Zhang et al. 2019). In this study, CcEG1 could also activate plant immunity, but it was not required for the pathogenicity of *C. chrysosperma*, which may result from gene duplication or redundancy because there were five GH12 proteins in *C. chrysosperma*. We need further analysis of other GH12 family proteins to reveal their functions during *C. chrysosperma*-plant interaction.

Conclusions

In conclusion, our findings showed that CcEG1 acts as an elicitor to trigger plant immune responses and interacts with receptor-like kinases *NbBAK1* and *NbRXEG1* to induce plant cell death in the apoplast, but it was not required for fungal virulence.

Methods

Microbial strains and plant growth conditions

The *C. chrysosperma* (CFCC_89981) wild-type and mutant strains were cultured and maintained on potato dextrose agar (PDA) media at 25°C in the dark. The *C. chrysosperma* used for DNA or RNA extraction was cultured in potato dextrose broth (PDB) at 25°C, 150 rpm for 2 days. *E. coli* DH5α was cultured in lysogeny broth (LB) at 37°C that used for plasmid construction. *Agrobacterium* strain GV3101 was cultured at 30°C and used for *agrobacterium*-mediated transient expression in *N. benthamiana* leaves. *N. benthamiana* was grown at 25°C and 80% relative humidity with 16 h photoperiod one day in the greenhouse. The medium used for hydrolytic

activity assay contains 2.5 g/L Na_2HPO_4, 2.5 g/L KH_2PO_4, 1.5 g/L peptone, 20 g/L agar, and 20 g/L glucose, PH = 7, and supplied with CMC-Na or cellulase (Sigma, USA), respectively.

Bioinformatics analysis

GH12 family members of *C. chrysosperma* were identified in its genome sequence database (the draft genome sequence had been sequenced by our lab.) with the protein sequence of XEG1 and other reported GH12 genes. The resulted hits were then confirmed the existence of GH12 domain by the Interpro (http://www.ebi.ac.uk/interpro/search/sequence/) and NCBI. The signal peptide was predicted by SignalP 5.0 (https://services.healthtech.dtu.dk/service.php - 5.0). The manually corrected members were arrayed by the ClustalW2 program, and phylogenetic dendrograms were constructed using PhyML implemented in SeaView with maximum likelihood (http://doua.prabi.fr/software/seaview) and MEGA11 was used to view the graph (www.megasoftware.net) (Gouy et al. 2010; Tamura et al. 2011). The N-glycosylation site was predicted in NetNGlyc (https://services.healthtech.dtu.dk/service.php?NetNGlyc-1.0). The phosphorylation site was predicted in NetPhos (https://services.healthtech.dtu.dk/service.php?NetPhos-3.1).

Plasmid construction and agroinfiltration assay

The tested fragments used to generate silencing or overexpression plasmids were amplified by PCR from cDNA or genomic DNA of *N. benthamiana* and *C. chrysosperma* using the specific primers (Additional file 3: Table S2), including *BAK1*, *CcEG1*, *CcEG1*-Δcbm1, and the variants that remove their signal peptides or replace with a plant signal peptide of PR-1. The CBM1 and SP-CBM1were obtained by synthesis. For transient expression in *N. benthamiana*, all of CcEG1, $CcEG1^{\Delta SP}$, $PR1^{SP}$-$CcEG1^{\Delta SP}$, CcEG1-Δcbm1, CBM1, and SP-CBM1 were separately cloned into the pGR106 and transformed into the *Agrobacterium tumefaciens* strain GV3101 by using the freeze–thaw method. The *BAX* and *GFP* were also cloned into pGR106 and used as positive and negative controls, respectively.

Virus-induced gene silencing (VIGS) assays in *N. benthamiana*

To silence the target gene in *N. benthamiana*, we constructed the plasmids of pTRV2:*BAK1* and pTRV2:*SOBIR1* and transformed into *A. tumefaciens* GV3101.

All the plasmids were sequenced and validated by Tsingke (Tsingke, China). Agroinfiltration assays were using bacterial cells resuspended in $MgCl_2$ buffer (10 mM $MgCl_2$, 10 mM MES, 200 μM acetosyringone, pH 5.6), adjusted to a final OD_{600} of 0.4, and stay in darkness for 5 h at 30°C and then injected in the back of *N. benthamiana* leaf. Each assay was performed on three individual plants, and repeated at least three times. All the primers used in this study were listed in Additional file 3: Table S2. The plasmid vectors pTRV1 and recombinant pTRV2 were transformed into *A. tumefaciens* GV3101, respectively. Then mixing the *A. tumefaciens* cell suspension carrying pTRV1 with *A. tumefaciens* cell suspension carrying pTRV2 vectors in a 1:1 ratio before infiltration, and infiltrated the mixed cell suspension into the *N. benthamiana* leaves. The leaves treated with *A. tumefaciens* carrying the pTRV1 and pTRV2: *GFP* were used as control. The silencing efficiency of the target gene was verified by qRT-PCR three weeks after agroinfiltration. The experiments were repeated three times.

Yeast signal sequence trap system

The secretory function of *CcEG1* signal peptide was substantiated by the yeast signal trap system (Jacobs et al. 1997). Briefly, the signal peptide of *CcEG1* was attached to the non-secretory invertase gene (SUC2) plasmid pSUC2 to construct pSUC2:$CcEG1^{SP}$, then transformed into the yeast strain YTK12 and cultured on CMD-W medium (lacking tryptophan). The positive transformants were identified by PCR with vector-specific primers (Additional file 3: Table S2) and incubated on YPRAA medium (2% raffinose). YTK12 transformed with pSUC2:$Avr1b^{SP}$ and the empty pSUC2 vector were used as positive and negative controls, respectively.

ROS activity and cellulose deposition assays

The accumulation of ROS was detected with 3, 3′-Diaminobenzidine (DAB) solution (Sigma, USA) after 24 h post inoculation (hpi) in *N. benthamiana* leaves, as described previously (Xu et al. 2022). Callose deposition was detected by aniline blue staining. Briefly, the agroinfiltrated *N. benthamiana* leaves were discolored with 95% ethanol and stained with 0.05% aniline blue in 0.067 M K_2HPO_4 buffer overnight, subsequently observed using a Leica DM2500 microscope (Leica, Weztlar, Germany).

Stress and pathogenicity assays

Cell wall-disruptor calcofluor white (CFW), H_2O_2, and Congo red (CR) were used to determine whether CcEG1 is involved in the response of external stress. 3-mm-diameter *C. chrysosperma* mycelial plugs was placed on the center of PDA agar plates containing 50 mg/mL CFW, 100 mg/mL CR, or 6% H_2O_2. Colony phenotypes were observed five days after inoculation. The experiment was performed three times independently.

For the pathogenicity assay, branches of *Populus tomentosa* were cut into 15-cm long and sealed both ends

by the wax. Then, the branches were scald by a 5 mm-diameter hot soldering iron and inoculated with *C. chrysosperma* mycelial plugs, maintaining in 25°C and wet environment. The lesions were photographed and measured at 4 dpi. The experiment was repeated three times.

Subcellular localization assay

To verify the subcellular localization of CcEG1, The *N. benthamiana* leaves were agroinfiltrated with CcEG1-pBinGFP4, $CcEG1^{\Delta SP}$-pBinGFP4, $PR1^{SP}$-$CcEG1^{\Delta SP}$-pBinGFP4, and the negative control pBinGFP4 for two days. The leaves were treated with water or 1 M NaCl, separately. Then cut into 8×8 mm^2 pieces and observed by the Laser confocal microscope (Leica SP8, Weztlar, Germany). The GFP fluorescence was excited using 488 nm laser line.

RNA extraction and RT-qPCR

Total RNA of *C. chrysosperma* and *N. benthamiana* leaves were extracted from the hypha grown in the PDB culture medium supplemented with poplar bark through RNA Easy Fast Plant Tissue Kit (TIANGEN, China) according to the manufacturer's instructions. First-strand cDNA was synthesized with 1 μg RNA by ABScript II cDNA First-stand Synthesis Kit (ABclonal, China). The following qPCR used the qPCR SYBR Green Master Mix (Yeasen, China). The genes *CcActin* and *NbActin* were used as endogenous controls in *C. chrysosperma* and *N. benthamiana*, separately. Relative expression levels were calculated by the $2^{-\Delta\Delta Ct}$ method with three independent biological replicates. All primers used in this study were listed in Additional file 3: Table S2.

Generation of deletion and complementation mutants

CcEG1 was knocked out by split-marker method combined with PEG-mediated protoplast transformation, as described previously (Natalie et al. 2003; Xu et al. 2022). All positive transformants were confirmed by PCR amplification with internal and external primers (Additional file 3: Table S2). The single copy homologous recombination event was identified by Southern blot. Briefly, the gDNA was digested by *EcoR* I and analyzed using the DIG High Prime DNA Labeling and Detection Starter Kit I, following the manufacturer's protocol (Roche, Germany).

For complementation, the whole *CcEG1* gene cassette, including ~ 1.5 kb native promoter, *CcEG1* genomic sequences, downstream 0.2-kb terminal sequences and geneticin-resistant cassette, were co-transformed into the protoplast of the deletion mutant. Then, transformants were selected by 30 μg/mL hygromycin and 50 μg/mL geneticin. Successful complementation was named as Δ*CcEG1*/C in this study. To verify the occurrence of a single copy homologous recombination event, the Southern blot analysis was performed with the DIG High Prime DNA Labeling and Detection Starter Kit I (Roche, Germany).

Protein extraction and Western blot

N. benthamiana leaves were collected and ground in liquid nitrogen at two days post inoculation for the extraction of total proteins by using plant protein extraction kit (BC3720; Solarbio, China) according to the manufacturer's instructions.

The proteins were separated using 10% sodium dodecyl sulphate polyacrylamide electrophoresis gels and transferred to polyvinylidene difluoride membranes. The membrane was blocked in TBST with 5% nonfat dry milk for one hour at room temperature. Then added the anti-HA (AE008; ABclonal, China) antibodies in 1:2000 dilution at 4°C overnight. Subsequently, the membranes were washed three times and then incubated with rabbit anti HA-Tag pAb (AE036, ABclonal, China) secondary antibodies in 1:5000 dilution. ECL chemiluminescence solution was used to detect HA antibody labeled with horseradish peroxidase (HRP).

Abbreviations

CBM	Carbohydrate-binding module
CFW	Calcofluor white
CR	Congo red
CWDEs	Cell wall-degrading enzymes
DAB	3, 3′-Diaminobenzidine
DPI	Days post inoculation
ET	Ethylene
ETI	Effector triggered immunity
H_2O_2	Hydrogen peroxide
HPI	Hours post inoculation
HRP	Horseradish peroxidase
JA	Jasmonic acid
LB	Lysogeny broth
LRR	Leucine-rich repeat
MM media	Minimal methanol medium
PAMPs	Pathogen-associated molecular patterns
PDA	Potato dextrose agar
PDB	Potato dextrose broth
PR1	Pathogenesis-related protein
PTI	PAMPs triggered immunity
RLKs	Receptor-like kinases
RLPs	Receptor-like proteins
SA	Salicylic acid
TRV	Tobacco rattle virus
VIGS	Virus-induced gene silencing

Supplementary Information

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Additional file 1: **Figure S1**. Subcellular localization of $CcEG1^{\Delta SP}$-GFP4 and pBINGFP4. $CcEG1^{\Delta SP}$-GFP4, CcEG1-GFP4, $PR1^{SP}$-$CcEG1^{\Delta SP}$-GFP4, and pBINGFP4 were transiently expressed in *N. benthamiana* and observed 24 hpi by Confocal Microscope SP8. The bars represent 50 μm. **Figure S2**. CcEG1 did not affect the growth and stress resistance of *C. chrysosperma*. The plugs of *C. chrysosperma* WT, Δ*CcEG1_14*, Δ*CcEG1_19*, and Δ*CcEG1/C*

strains were placed on the PDA plates with 50 mg/mL CFW or 100 mg/mL CR, or inoculated their conidia into PDA plates with 6% H_2O_2 at 25°C for four days. This experiment was performed three times with similar results, and each assay was performed on at least three independent biological repeats. **Figure S3**. CBM1 was unable to induce plant immune defense response in *N. benthamiana*. Determining the relative expression of HR and PTI marker genes 24 hours after SP-CBM1 was inoculated into *N. benthamiana* leaves. **Figure S4**. GH12 family members were widely distributed in both fungi and oomycetes. **a** Large numbers of GH12 family genes were existing in pathogenic fungi and oomycetes, and some of them contained CBM1 domain. **b** Phylogenetic tree of 58 GH12 family genes from different phytopathogens, green pentacle means that they are able to induce plant cell death.

Additional file 2: **Table S1**. GH12 members in different species.

Additional file 3: **Table S2**. Primers used in this study.

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Author contributions
ZX, ZL, and MG performed the experiment, ZX and DX analyzed the data and wrote the manuscript. CT and DX designed the research. All authors read and approved the final manuscript.

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Declarations

Ethics approval and consent to participate
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Consent for publication
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Competing interests
The authors declare that they have no competing interests.

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