

RESEARCH ARTICLE

Open Access



# Characterization of two SGNH family cell death-inducing proteins from the horticulturally important fungal pathogen *Botrytis cinerea* based on the optimized prokaryotic expression system

Xiaokang Zhang<sup>1,2</sup>, Zhanquan Zhang<sup>1,2</sup>, Tong Chen<sup>1</sup>, Yong Chen<sup>1</sup>, Boqiang Li<sup>1</sup> and Shiping Tian<sup>1,2\*</sup> 

## Abstract

*Botrytis cinerea* is one of the most destructive phytopathogenic fungi, causing significant losses to horticultural crops. As a necrotrophic fungus, *B. cinerea* obtains nutrients by killing host cells. Secreted cell death-inducing proteins (CDIPs) play a crucial role in necrotrophic infection; however, only a limited number have been reported. For high-throughput CDIP screening, we optimized the prokaryotic expression system and compared its efficiency with other commonly used protein expression systems. The optimized prokaryotic expression system showed superior effectiveness and efficiency and was selected for subsequent CDIP screening. The screening system verified fifty-five candidate proteins and identified two novel SGNH family CDIPs: BcRAE and BcFAT. *BcRAE* and *BcFAT* exhibited high expression levels throughout the infection process. Site-directed mutagenesis targeting conserved Ser residues abolished the cell death-inducing activity of both BcRAE and BcFAT. Moreover, the transient expression of BcRAE and BcFAT in plants enhanced plant resistance against *B. cinerea* without inducing cell death, independent of their enzymatic activities. Our results suggest a high-efficiency screening system for high-throughput CDIP screening and provide new targets for further study of *B. cinerea*-plant interactions.

**Keywords** *Botrytis cinerea*, Cell death-inducing protein, SGNH family hydrolases, Plant-fungus interaction

## Core

In this study, we optimized the prokaryotic expression system for screening the cell death-inducing protein (CDIP). The optimized screening system identified two novel CDIPs in the SGNH family of *B. cinerea*, namely BcFAT and BcRAE. Sufficient BcFAT and BcRAE can

trigger plant cell death, which depends on their enzymatic activities. The transient expression of BcFAT and BcRAE in plants was found to induce plant resistance against *B. cinerea* without inducing cell death, which is independent of enzyme activity.

## Gene and accession numbers

Information for the genes in this article can be found in the National Center for Biotechnology Information (NCBI) database, accession numbers: BcFAT (BCIN\_07g03280), BcRAE (BCIN\_02g07100).

\*Correspondence:

Shiping Tian  
tsp@ibcas.ac.cn

<sup>1</sup> Key Laboratory of Plant Resources, Institute of Botany, Chinese Academy of Sciences, Beijing 100093, China

<sup>2</sup> University of Chinese Academy of Sciences, Beijing 100049, China



© The Author(s) 2024. **Open Access** This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit <http://creativecommons.org/licenses/by/4.0/>. The Creative Commons Public Domain Dedication waiver (<http://creativecommons.org/publicdomain/zero/1.0/>) applies to the data made available in this article, unless otherwise stated in a credit line to the data.

## Introduction

*Botrytis cinerea*, the causal agent of the notorious plant disease gray mold, is one of the most devastating plant pathogens worldwide. *B. cinerea* has a remarkably wide host range and infects more than 1000 plant species, including almost all fruit and vegetable crops (Yigal et al. 2016). Moreover, *B. cinerea* attacks horticultural crops not only in the field but also during postharvest storage, causing an annual economic loss of over \$10 billion (Chen et al. 2023b). Due to its substantial economic impact and scientific importance, *B. cinerea* ranks second among the top ten plant fungal pathogens worldwide (Dean et al. 2012). Currently, the control of *B. cinerea* is still mainly dependent on chemical fungicides in agricultural production. However, fungicide-resistant strains have emerged in the field, and the overuse of fungicides has led to increasing concerns about environmental pollution and chemical residues (Poveda et al. 2020). Thus, alternative strategies are urgently needed to control *B. cinerea*.

Bio-sourced antifungal substances have attracted broad attention recently for their prominent properties, such as being non-toxic, readily available, and environmentally friendly (Chen et al. 2023a). To date, a series of bio-sourced substances have been reported with remarkable antifungal activities and promising potential for controlling *B. cinerea* (Wang et al. 2020; Cui et al. 2021; Sun et al. 2021; Li et al. 2022a; Zhang et al. 2023). An in-depth understanding of the pathogenic mechanism of *B. cinerea* is conducive to discovering novel antifungal substances and promoting the practical application of biogenic substances. In addition, controlling essential pathogenic factors or inducing disease resistance in postharvest fruits or vegetables are alternative strategies to reduce the dependence on chemical fungicides, which also requires an in-depth understanding of the pathogenic mechanisms of *B. cinerea* and the *B. cinerea*-plant interaction mechanisms (An et al. 2015; Li et al. 2020; Gu et al. 2021; Li et al. 2022b; Lu et al. 2023; Yang et al. 2023). Therefore, a deep understanding of the pathogenic mechanism of *B. cinerea* is of great significance for developing effective control strategies.

*B. cinerea* has been reported to employ multiple mechanisms to promote necrotrophic infection, such as the release of phytotoxic metabolites as well as the secretion of cell wall-degrading enzymes and cell death-inducing proteins (CDIPs) (Velo and van Kan 2018; Zhang et al. 2021a). Among them, CDIPs trigger hypersensitive response-like cell death in plants, and the modes of action of several CDIPs have been elucidated. For example, SPL1, an abundantly secreted cerato-platanin family protein, interacts with plant PR1 and induces cell death in plants (Zhang et al. 2017; Yang et al. 2018a). The abundantly secreted glycoprotein BcIEB1 was reported to interact with PR5 and trigger plant cell death, possibly as a pathogen-associated mode

(González et al. 2017). The xyglucanase BcXYG1 causes intense plant necrosis through a SOBIR1/BAK1-related pathway, and its cell death-inducing activity is independent of its xyglucanase activity (Zhu et al. 2017). The recently reported BcCrh1 is secreted into the plant apoplast and translocated into the plant cell, triggering cell death and the immune response (Bi et al. 2021). These results indicate that CDIPs activate the plant immune system and trigger cell death critical to infection. However, only a few CDIPs have been reported among more than 500 secreted proteins of *B. cinerea* (Zhu et al. 2017, 2022; Yang et al. 2018b; Bi et al. 2021; Jeblick et al. 2023). Recent research has shown that the secretome of multiple knockout mutants generated by CRISPR/Cas9 that lack 12 reported CDIPs retains substantial phytotoxic activity, suggesting that there are still unknown CDIPs contributing to necrosis and virulence (Leisen et al. 2022). To further identify novel CDIPs, an efficient high-throughput characterization system is necessary. Prokaryotic, eukaryotic, and *Agrobacterium*-mediated transient expression systems are three commonly used heterologous protein expression systems. *Agrobacterium*-mediated transient expression system is a widely used CDIP screening and characterization system. However, unstable protein expression efficiency and unquantifiable protein expression levels may affect the screening and characterization of CDIPs. The prokaryotic expression system has a high protein expression level and efficiency. However, the frequent formation of insoluble inclusion bodies of prokaryotic expression limits its expression effect and application. The eukaryotic expression system has post-translational protein modifications with low interference from protein impurities. Nevertheless, which system is more suitable for screening and characterizing CDIPs has not yet been compared.

Here, we optimized the prokaryotic protein expression system, promoted the formation of soluble proteins, and established a CDIP screening system based on the optimized prokaryotic expression. We further compared the screening efficiency and effectiveness of three protein expression systems. The optimized prokaryotic expression system exhibited superior screening effects and was selected as the final screening system. With the help of the screening system, we screened CDIPs from 55 candidate proteins and identified two novel SGNH family CDIPs. The SGNH superfamily is a large hydrolase family named after four highly conserved residues (serine, glycine, asparagine, and histidine) and comprises a diverse range of carbohydrate-modifying enzymes (Anderson et al. 2022; Lescic Asler et al. 2017). SGNH family members have been reported to contribute to plant immunity (Oh et al. 2005; Kwon et al. 2009; Kim et al. 2013). However, there are few reports on the role of fungal SGNH proteins in plant-fungus interactions. Therefore, we also conducted preliminary functional studies on these two SGNH family CDIPs in this study.

## Results

### Optimization of the prokaryotic expression system

Most *B. cinerea*-secreted proteins have small molecular weights and are prone to form insoluble inclusion bodies during prokaryotic expression. To optimize the prokaryotic expression system for CDIP screening, we explored the expression effects of different expression vectors and expression conditions. The results showed that the CDIPs expressed by the pET30a vector at 37 °C and 15 °C were only present in the sediment (Fig. S1A and B), indicating that the protein formed insoluble inclusion bodies under these expression conditions. With the help of the pCold-TF vector harboring the molecular chaperone trigger factor (TF), we induced the expression of TF-CDIP recombinant proteins at 15 °C overnight. The TF-CDIPs mainly existed in the supernatant and existed in the precipitation (Fig. S1C). After further purification of the supernatant, we obtained TF-CDIP recombinant proteins with prominent bands (Fig. 1A). Moreover, in the subsequent protein expression, most proteins were present in the supernatant (Fig. S2). To further verify the effect of TF on plants, we infiltrated tobacco with different concentrations of TF. The results showed that 20–200  $\mu$ M TF had no obvious impact on plants (Fig. 1B). These results indicate that overnight induction at 15 °C with the help of the molecular chaperone TF is a suitable condition for prokaryotic expression of CDIP.

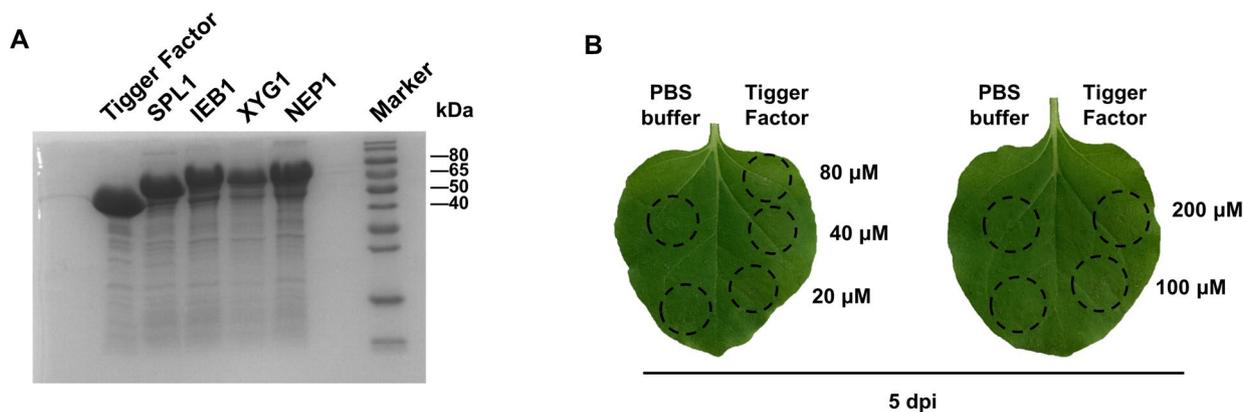
### Comparison of protein-expressing systems for screening CDIPs

High-throughput screening of CDIPs requires an effective and efficient screening system. To determine the appropriate screening system, we compared the efficiency and effectiveness of the eukaryotic, prokaryotic, and *Agrobacterium*-mediated transient expression systems on CDIP

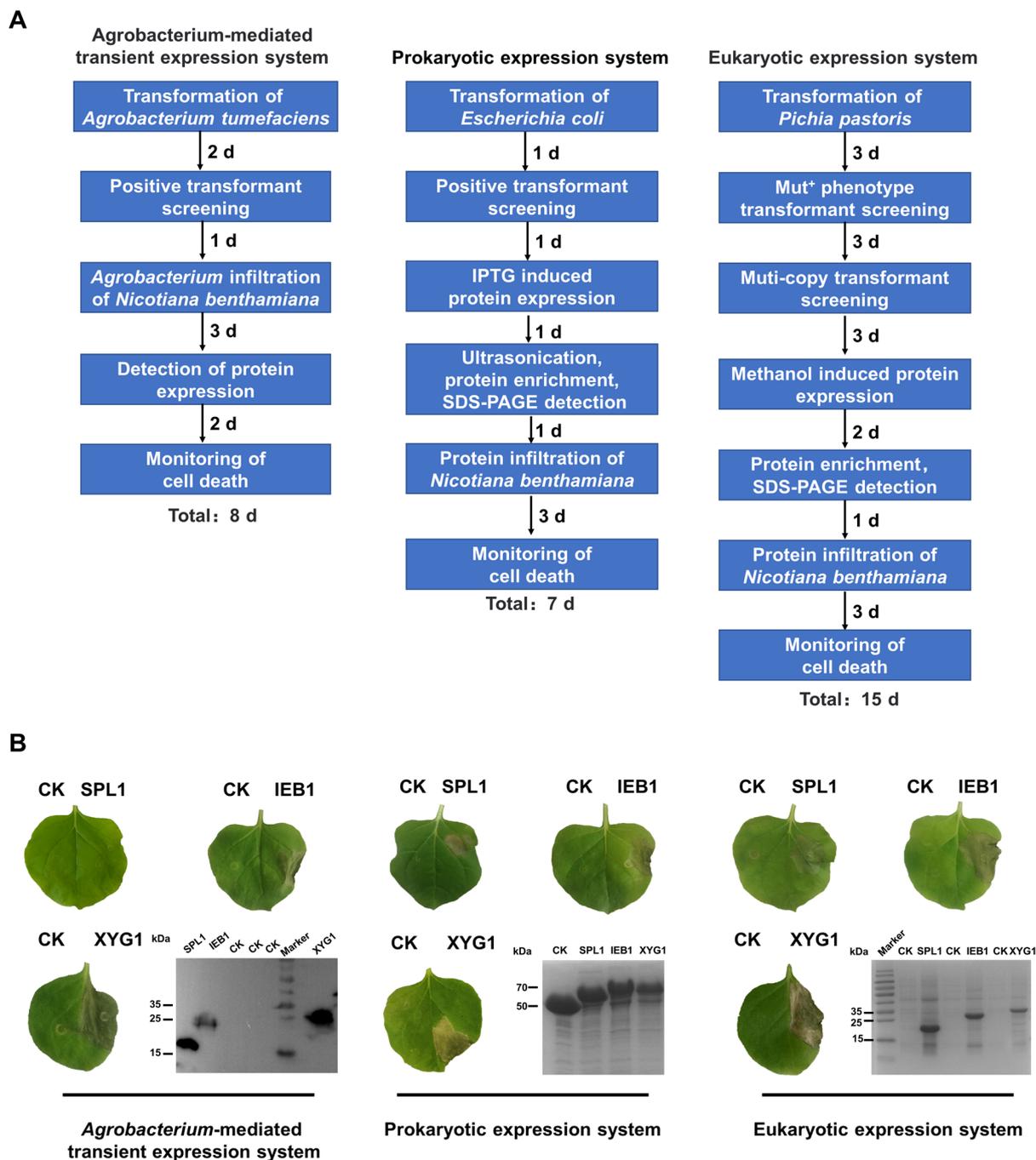
screening. Previously reported CDIPs, including BcSPL1 (Frías et al. 2011), BcXYG1 (Zhu et al. 2017), and BcIEB1 (Frías et al. 2016), were expressed by different protein expression systems. The necrosis-inducing activities were then verified in tobacco leaves. Figure 2A shows the protein expression processes of the three protein expression systems. Compared with prokaryotic and *Agrobacterium*-mediated transient expression systems, the eukaryotic system is time-consuming and labor-intensive. As shown in Fig. 2B, all proteins expressed by the eukaryotic and prokaryotic expression systems induced necrosis in plants. However, the transiently expressed BcSPL1 failed to cause necrosis, and the WB results showed that the protein was successfully expressed in plants. The above results suggest that eukaryotic and prokaryotic expression systems are more effective than the transient expression system for CDIP screening. The *Agrobacterium*-mediated transient expression system is a commonly used screening system for necrosis-inducing proteins. Our results indicate that the transient expression system probably misses important CDIPs during screening. Moreover, the eukaryotic expression system is time-consuming and labor-intensive, thus unsuitable for high-throughput screening. The prokaryotic expression system exhibited outstanding screening efficacy, high expression levels, and low time and labor costs. Therefore, the prokaryotic expression system was chosen for the subsequent screening of CDIPs.

### Screening of CDIPs by the optimized prokaryotic expression system

The flowchart of screening CDIPs with the optimized prokaryotic expression system is shown in Fig. 3A. With the help of the optimized prokaryotic expression system, we



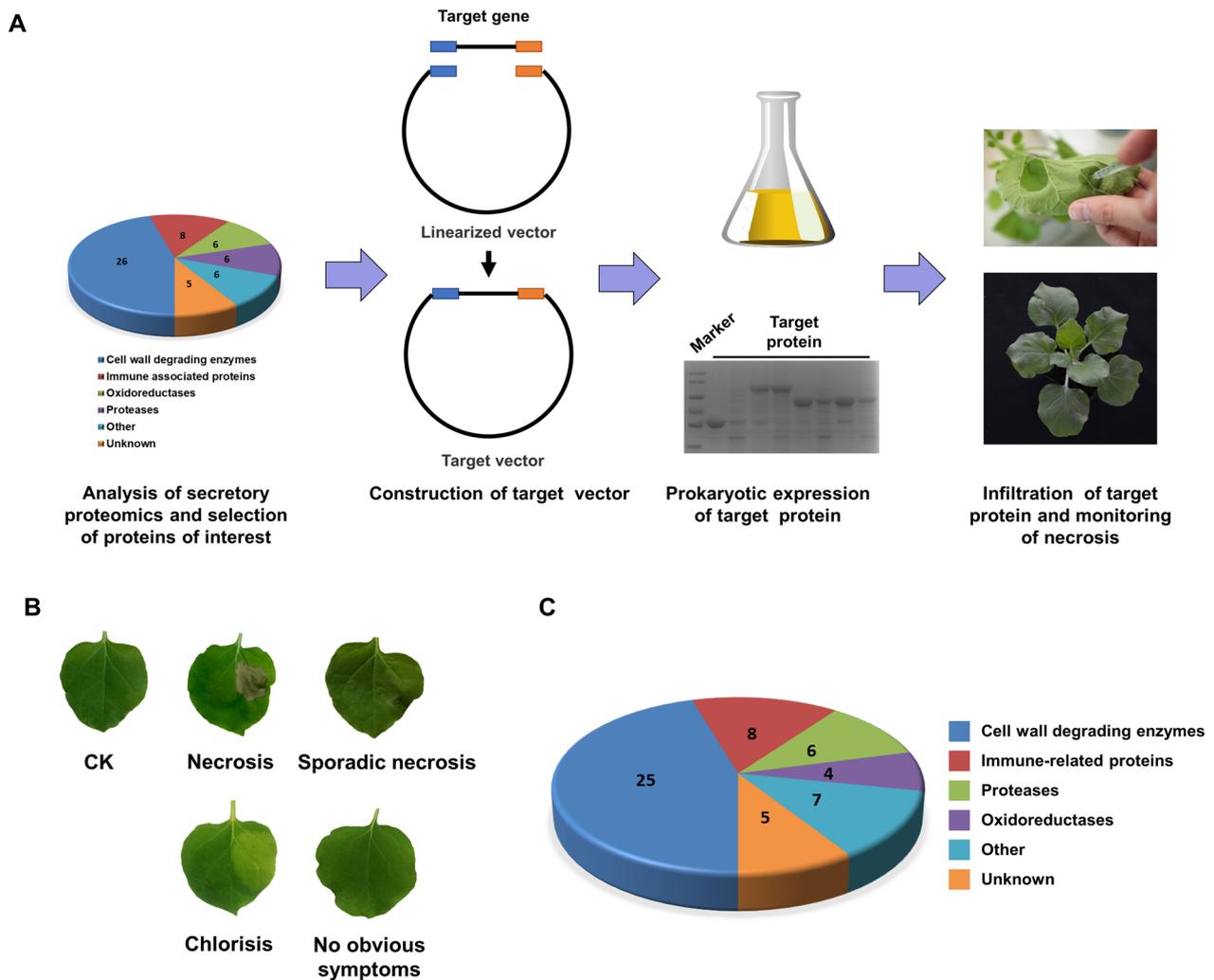
**Fig. 1** Optimization of the prokaryotic expression system. **A** Expression of soluble recombinant cell death-inducing proteins (CDIPs) utilizing the optimized prokaryotic expression system. The reported CDIPs BcIEB1 (BCIN\_15g00100), BcNEP1 (BCIN\_06g06720), BcSPL1 (BCIN\_03g00500), and BcXYG1 (BCIN\_03g03630) were recombinantly expressed with trigger factor (TF) using the prokaryotic expression system, and the TF was expressed as a control. The supernatant of disrupted cells was used for protein purification. **B** The impact of TF on plants. TF at different concentrations were infiltrated into tobacco leaves, and 10 mM phosphate-buffered saline (PBS) was used as the negative control. Photos were taken 5 d after infiltration. Black dashed circles indicate infiltrated sites



**Fig. 2** Comparison of different protein expression systems. **A** Flow chart of the protein expression processes of different systems. **B** The reported CDIPs BciIEB1, BcSPL1, and BcXYG1 were used to test the screening effects of different expression systems. *Agrobacterium tumefaciens* for transient expression was infiltrated into tobacco leaves at OD600=0.5. Photos were taken five days after infiltration. Western blotting (WB) was performed to verify the expression of proteins in plants. SDS-PAGE verified the proteins expressed by the eukaryotic and prokaryotic expression systems. Proteins expressed by the eukaryotic and prokaryotic expression systems were infiltrated into tobacco leaves at a concentration of 50 μM. Photos were taken 3 d after infiltration. Empty vectors were used to express control proteins (CK), respectively

further screened CDIPs of *B. cinerea*. We first selected 55 candidate proteins from the reported secretomes. Detailed information about the candidate proteins, including the gene

ID number, amino acid number, function, and signal peptide, is shown in Table S1. The candidate proteins were expressed through the prokaryotic expression system, and proteins

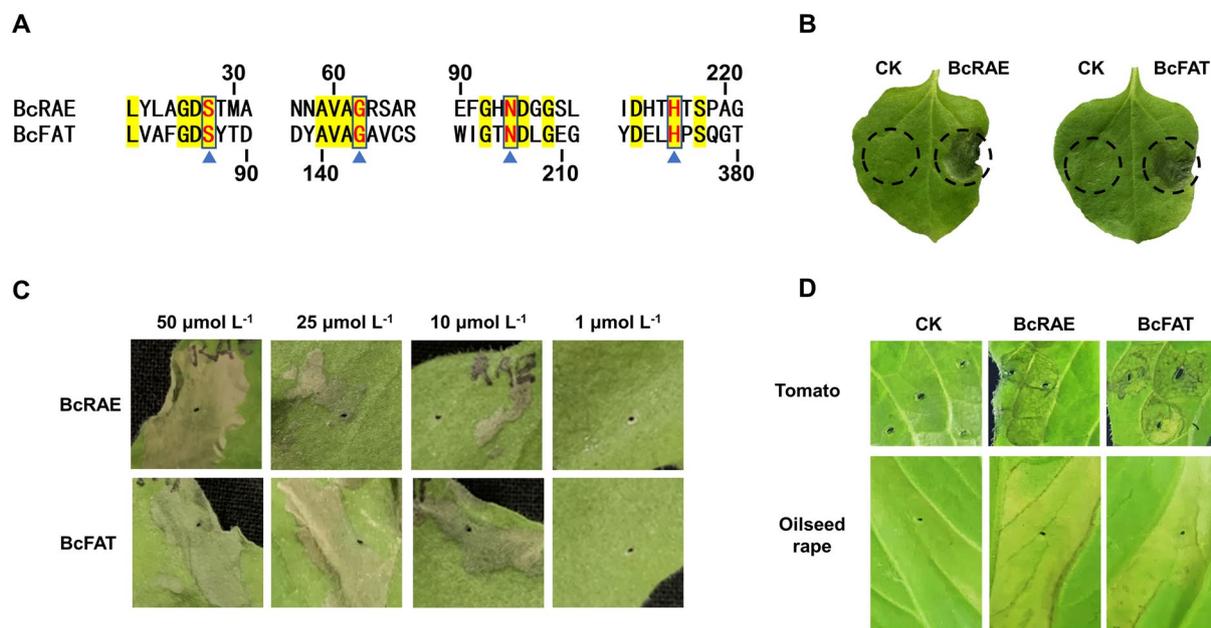


verified by SDS-PAGE (Fig. S2) were infiltrated into *Nicotiana benthamiana* leaves to detect cell death symptoms. The cell death symptoms were classified into necrosis, sporadic necrosis, chlorosis, and no obvious necrosis. Representative leaves are shown in Fig. 3B. The candidate proteins were classified by their functions, mainly into cell wall-degrading enzymes, immune-related proteins, oxidoreductases, proteases, and other proteins of unknown function (Fig. 3C).

#### Two SGNH hydrolases, BcRAE and BcFAT, lead to cell death in plants

By screening candidate proteins, two novel CDIPs, rhamnogalacturonan acetyltransferase (BcRAE, BCIN\_02g07100)

and fatty acyltransferase (BcFAT, BCIN\_07g03280), were identified. The functional analysis of BcRAE and BcFAT showed that they belong to the SGNH hydrolase superfamily. The conserved residues of BcRAE and BcFAT are marked in Fig. 4A. As shown in Fig. 4B, BcRAE and BcFAT induced necrosis in tobacco leaves 3 d after infiltration, while CK protein had no apparent impact. Therefore, BcRAE and BcFAT were chosen for further mechanism research. To investigate the effect of protein concentration on the cell death-inducing activities of BcRAE and BcFAT, we adjusted the protein concentration to 1–50  $\mu$ M followed by tobacco leaf infiltration. The results showed that 50  $\mu$ M BcRAE caused intense



**Fig. 4** BcRAE and BcFAT induced cell death in plants. **A** Protein sequence alignment of BcRAE and BcFAT conserved motifs. Identical residues are highlighted with a yellow background. Red letters marked with blue triangles indicate the conserved residues Ser, Gly, Asn, and His in the SGNH hydrolases. The numbers indicate the position of the amino acid in the protein sequence. **B** Cell death symptoms of tobacco caused by BcRAE or BcFAT. Tobacco leaves were infiltrated with 50  $\mu\text{M}$  BcRAE or BcFAT. Photos were taken 3 d after infiltration. Black dashed circles indicate infiltrated sites. **C** Effect of protein concentration on the cell death-inducing activities of BcRAE and BcFAT. For each treated leaf, the protein-infiltrated area was similar in size. Symptoms were photographed 5 d after infiltration. **D** Effect of BcRAE and BcFAT on tomato and oilseed rape leaves. Tomato and oilseed rape leaves were infiltrated with 50  $\mu\text{M}$  proteins. Images were taken 5 d after infiltration

necrosis. When the protein concentration was reduced to 25  $\mu\text{M}$  and 10  $\mu\text{M}$ , the cell death symptoms gradually weakened, and 1  $\mu\text{M}$  BcRAE failed to cause cell death. BcFAT, at concentrations of 50 and 25  $\mu\text{M}$ , induced intense necrosis. When the concentration was low to 10  $\mu\text{M}$ , the necrosis symptoms were weakened, and 1  $\mu\text{M}$  BcRAE failed to cause cell death (Fig. 4C). Moreover, we investigated the effect of BcRAE and BcFAT on tomato and oilseed rape leaves. As shown in Fig. 4D, BcRAE and BcFAT also triggered cell death in tomato and oilseed rape leaves. These results indicate that BcRAE and BcFAT can induce cell death in various plants, and the cell death-inducing activities of BcRAE and BcFAT are associated with protein concentration.

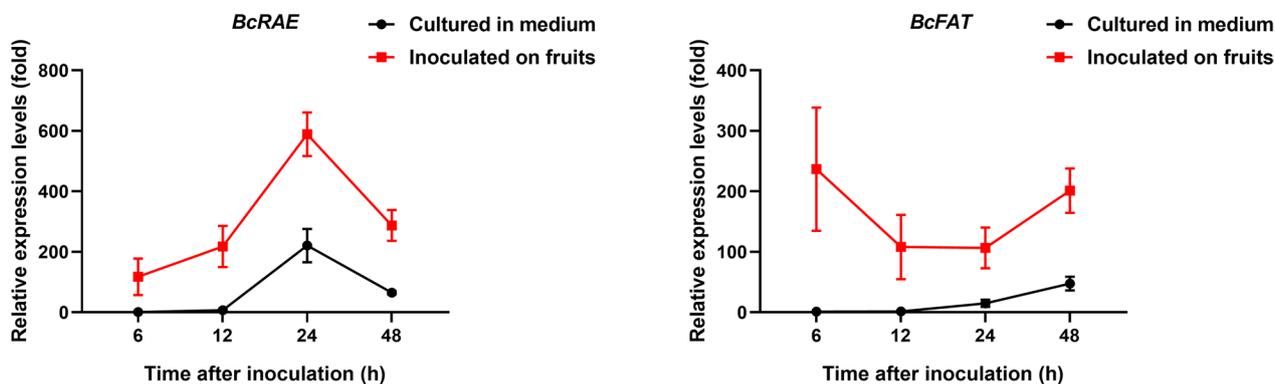
#### BcRAE and BcFAT are highly expressed during infection

To investigate the expression patterns of *BcRAE* and *BcFAT*, we performed qRT-PCR to detect the relative expression levels of *BcRAE* and *BcFAT* at various time points during *B. cinerea* development and infection. As shown in Fig. 5, during the development of *B. cinerea* cultured in the medium, the expression level of *BcFAT* gradually increased, and the expression level of *BcRAE* peaked at 24 h post-inoculation (hpi) and then decreased. Moreover, both *BcRAE* and *BcFAT* exhibited

elevated expression levels throughout the infection process (Fig. 5). Specifically, the expression of *BcRAE* during infection showed a similar trend to that during culture. On the other hand, the expression of *BcFAT* during infection increased sharply at 6 hpi, then gradually weakened during 6–24 hpi, and rose during 24–48 hpi. These results indicate that the expression of both *BcFAT* and *BcRAE* is induced during infection and suggest the potential involvement of *BcRAE* and *BcFAT* in the infection process of *B. cinerea*.

#### BcRAE and BcFAT induce cell death depending on their enzymatic activities

The SGNH hydrolase catalyzes the cleavage of the ester bonds, in which the conserved Ser residue is the nucleophile and proton donor. The Ser residue also participates in the formation of oxyanion holes and the catalytic triad, so it plays a crucial role in catalysis (Mlgaard et al. 2000; Akoh et al. 2004; Lescic Asler et al. 2017). To test whether the conserved Ser residues of BcRAE and BcFAT are needed for the induction of necrosis, we performed site-directed mutagenesis of Ser residues to Ala. Residues Ser28 of BcRAE and Ser87 of BcFAT were mutated to Ala. The mutant proteins were expressed in *E. coli* (Fig. 6A) and infiltrated into tobacco leaves. The CK and



**Fig. 5** *BcRAE* and *BcFAT* are highly expressed during infection. *B. cinerea* conidia were inoculated on tomato fruits and in PDB medium. Samples were collected 6, 12, 24, and 48 h after inoculation. qRT-PCR analysis was performed to measure the expression levels of *BcRAE* and *BcFAT*. *BctubA* (BCIN\_01g08040) served as the reference gene, and the gene expression level of *B. cinerea* cultured in PDB for 6 h was set to 1. The data, presented as the mean  $\pm$  standard deviation (SD), represent three independent experiments

wild-type BcRAE and BcFAT proteins served as negative and positive controls, respectively. As shown in Fig. 6B, the cell death activities of the mutant proteins BcRAE-S28A and BcFAT-S87A were abolished. We also investigated the effect of high-temperature denaturation on the cell death-inducing activities of BcFAT and BcRAE. The result showed that high-temperature denaturation abolished the cell death-inducing activities of both BcFAT and BcRAE (Fig. 6C). These results indicate that the induction of cell death by BcRAE and BcFAT depends on their enzymatic activities.

#### Knockout of BcRAE and BcFAT does not affect the growth and virulence of *B. cinerea*

To investigate the effects of BcRAE and BcFAT on the development and virulence of *B. cinerea*, we generated single and double knockout mutants of *BcRAE* and *BcFAT* ( $\Delta BcRAE-1$ ,  $\Delta BcRAE-2$ ,  $\Delta BcFAT-1$ ,  $\Delta BcFAT-2$ ,  $\Delta BcRAE/BcFAT-1$  and  $\Delta BcRAE/BcFAT-2$ ). No significant difference was found in the colony morphology and growth rate between the mutants and the wild type (Fig. S3A). In addition, there was no significant difference in virulence between the mutant and the wild type (Fig. S3B). These results suggest that there may be functional redundancy of BcRAE and BcFAT with other SGNH family members or CDIPs in *B. cinerea*.

#### BcRAE and BcFAT induce resistance of plants to *B. cinerea* independent of their enzymatic activities

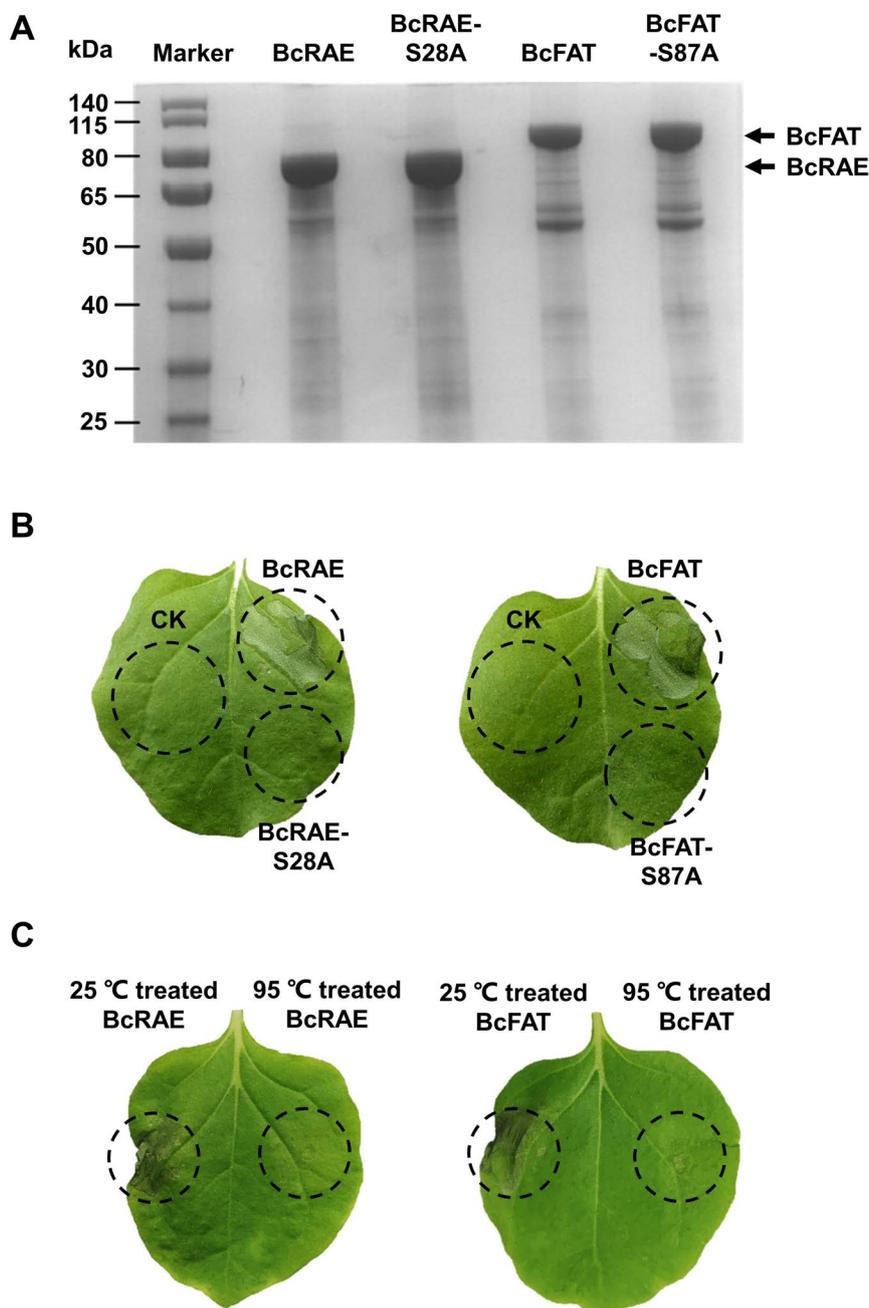
Previous studies have shown that some SGNH family esterases/lipases in plants have different regulatory effects on plant immunity (Oh et al. 2005; Hong et al. 2008; Kwon et al. 2009; Lee et al. 2009; Kim et al. 2013; Gao et al. 2017). To determine whether BcRAE and BcFAT induce plant immune responses, we transiently expressed BcRAE and

BcFAT by infiltrating *Agrobacterium* into tobacco leaves (no obvious symptoms, data not shown). WB results verified the expression of proteins in tobacco (Fig. 7A). We further inoculated *B. cinerea* spores on infiltrated sites three days after infiltration. The results showed that the lesion sizes of the leaves pretreated with BcRAE and BcFAT were significantly smaller ( $P < 0.05$ ) than those of the control (Fig. 7A and B). Moreover, BcFAT-pretreated tobacco leaves had smaller lesion sizes after inoculation with *B. cinerea* spores resuspended in PDB diluted 1:4 with water compared to control and BcRAE. In addition, we transiently expressed site-mutated BcFAT and BcRAE in tobacco. Interestingly, the transient expression of site-mutated proteins still significantly enhanced plant resistance to *B. cinerea* (Fig. 7C and D). These results indicate that BcRAE and BcFAT induce plant resistance to *B. cinerea* independent of their enzymatic activities, which differs from their cell death-inducing activities.

To further analyze the effect of BcFAT and BcRAE on defense-related genes, we infiltrated tobacco leaves with 50  $\mu$ M BcFAT, BcRAE, and CK protein. The expression levels of *NbPR1a*, *NbPR1b*, *NbPTIS*, and *NbRBOHB* at 24 hpi were detected by RT-qPCR. The results showed that BcRAE significantly induced the expression of *NbPR1b*, *NbPTIS*, and *NbRBOHB* but inhibited the expression of *NbPR1a*. BcFAT induced the expression of *NbPTIS* and *NbRBOHB* but had no significant effect on *NbPR1a* and *NbPR1b* (Fig. 7E). These results suggest that BcRAE and BcFAT induce plant immunity-related gene expression and enhance tobacco resistance to *B. cinerea*.

#### Discussion

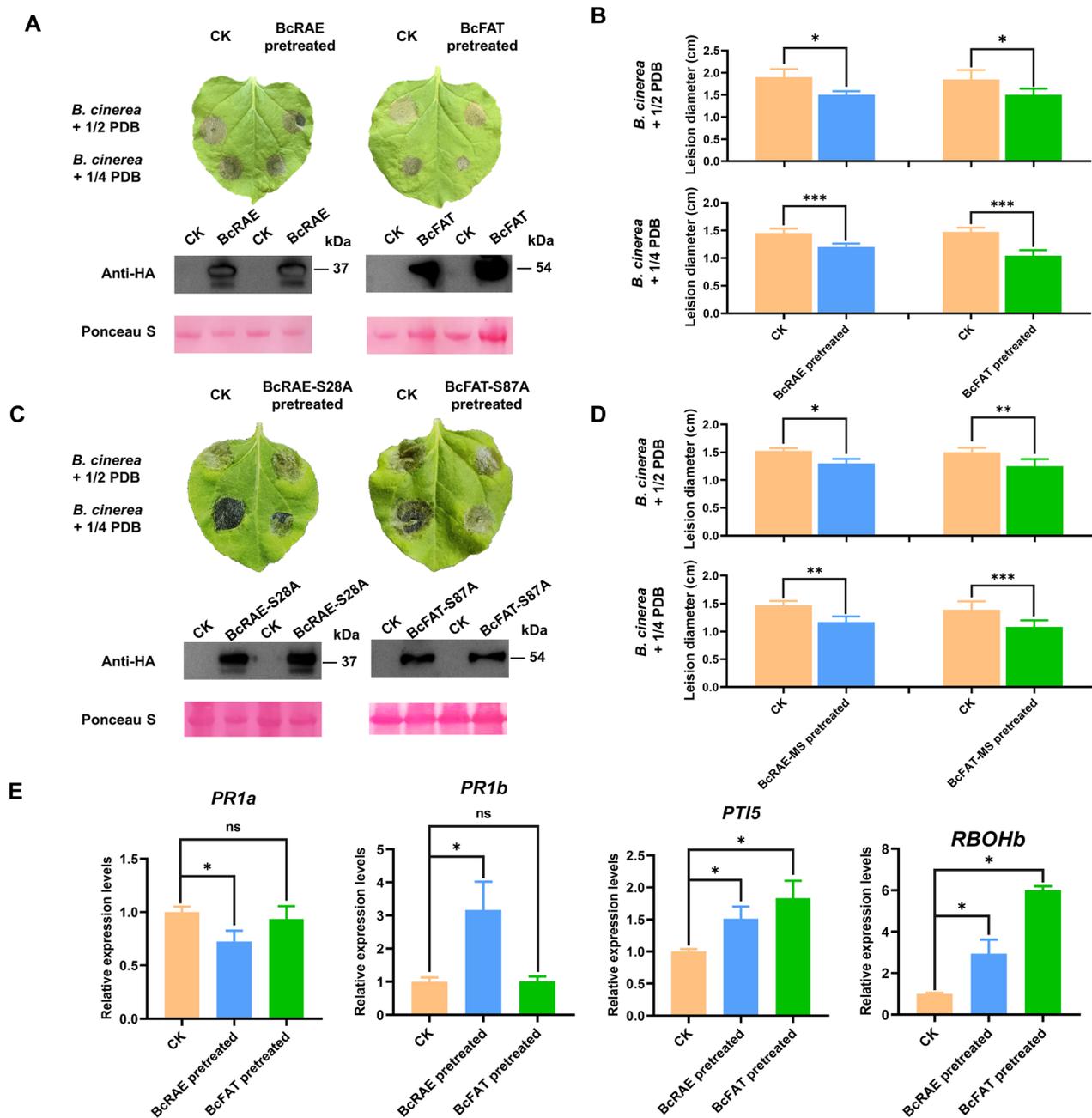
Unlike biotrophic pathogens that absorb nutrients from living cells, necrotrophic pathogens generally kill host cells to obtain nutrients. As a necrotrophic fungus,



**Fig. 6** BcRAE and BcFAT induce cell death depending on SGNH hydrolase activity. **A** SDS-PAGE analysis of native and mutant proteins. The positions of the target proteins were marked with black arrows. **B** Treatment of tobacco leaves with 50  $\mu$ M CK, native BcRAE and BcFAT, and mutant proteins. BcRAE-S28A: the Ser28 residue of BcRAE was mutated to Ala. BcFAT-S87A: the Ser87 residue of BcFAT was mutated to Ala. Photos were taken 3 d after treatment. Black dashed circles indicate infiltrated sites. **C** Effect of high-temperature denaturation on the cell death-inducing activities of BcFAT and BcRAE. Prokaryotically expressed proteins were incubated at 95 °C for 15 min and infiltrated into tobacco leaves, and proteins treated at 25 °C were infiltrated as the control. Photos were taken 3 d after infiltration. Black dashed circles indicate infiltrated sites

*B.cinerea* has long been thought to kill hosts crudely by secreting numerous cell wall-degrading enzymes, toxins, and other metabolites. However, recent studies have shown that the interaction between *B. cinerea* and host

plants is complex and delicate (Bi et al. 2023). Once *B. cinerea* is in contact with host cells, they first face the trouble of obtaining nutrients from living cells and withstanding plant defense. Therefore, the early stage of



**Fig. 7** BcRAE and BcFAT induce resistance in *N. benthamiana*. **A** and **C** Phenotypes of plant resistance to *B. cinerea* induced by transiently expressed proteins. *BcFAT*, *BcRAE* **A** and their site-mutated proteins **C** were transiently expressed in *N. benthamiana*, and the *Agrobacterium* harboring the empty PVX vector was used as the control. The treated tobacco was kept in the culture room for three days, followed by inoculation of *B. cinerea* spores resuspended in PDB of different dilutions on the *Agrobacterium*-infiltrated site. Protein expression was detected by WB, and Ponceau S staining served as the loading control. The inoculated plants were kept in a humid chamber at 22 °C. Photographs were taken 60 h after inoculation. Each assay contained at least nine leaves, and the experiment was repeated three times. **B** and **D** Statistics of colony diameters. Colony diameters of *B. cinerea* grown on BcFAT, BcRAE **B** and their site-mutated proteins **D** pretreated tobacco were measured 60 h after inoculation. Data from three independent experiments are presented as the mean  $\pm$  SD. Asterisks represent a significant difference (Tukey test, \*,  $P < 0.05$ , \*\*,  $P < 0.01$ , \*\*\*,  $P < 0.001$ ); **E** *N. benthamiana* leaves were infiltrated with 50  $\mu$ M of control protein, *BcFAT*, and *BcRAE*. After 24 h of growth in the culture room, the treated leaves were picked for RNA extraction. qRT-PCR was used to analyze the expression levels of *NbPR1a*, *NbPR1b*, *NbPTI5*, and *NbRBOHb*. *NbE1a* was used as the reference gene. Data from three independent experiments are presented as the mean  $\pm$  SD. Asterisks represent a significant difference. (Tukey test,  $P < 0.05$ )

infection is critical for successful colonization. According to the infection model proposed by Shlezinger et al. (2011), the anti-apoptotic machinery protects *B. cinerea* from host-induced cell death, and *B. cinerea* secretes various necrosis-inducing factors to establish small infection zones. The surviving fungal cells develop and accumulate sufficient fungal biomass for further lesion expansion. Nevertheless, studies of *Sclerotinia sclerotiorum*, the sister species of *B. cinerea*, suggested a short biotrophic phase in its early infection stage (Kabbage et al. 2013). Veloso and van Kan (2018) proposed an alternative model. According to this model, the balance between apoptosis and autophagy in *B. cinerea* and the plant host determines the result of colonization. In the biotrophic phase, *B. cinerea* produces autophagy-suppressing molecules to suppress host autophagic cell death. When fungi accumulate sufficient biomass in host tissues, plant apoptosis triggered by fungal CDIPs and toxins replaces the suppression of autophagy, resulting in the death of plant tissues and the spread of disease.

Although whether the biotrophic phase is included in the early stages of *B. cinerea* infection remains unclear, a consensus exists between the two models that CDIPs play an essential role in the early stage of colonization. Therefore, the study of CDIPs is of great significance for analyzing the interaction between *B. cinerea* and plants. Previously reported secretomes of *B. cinerea* showed that more than 500 proteins are secreted during infection, including CWDEs, proteases, and many proteins with unknown functions (Shah et al. 2009a; Espino et al. 2010; Zhu et al. 2017). However, only a few CDIPs have been reported (Zhu et al. 2017, 2022; Yang et al. 2018b; Bi et al. 2021; Jeblick et al. 2023). Recently, Leisen et al. (2022) generated multiple knockout mutants lacking 12 reported CDIPs through the CRISPR/Cas9 system and found that the secretome of the mutant strains still retained substantial phytotoxic activity, indicating that there are still unknown CDIPs triggering cell death. Therefore, it is necessary to further screen CDIPs from the remaining large number of secreted proteins, and an efficient screening system is needed for high-throughput CDIP screening.

There are three commonly used protein expression systems: prokaryotic, eukaryotic, and *Agrobacterium*-mediated transient expression systems. The *Agrobacterium*-mediated transient expression system is convenient and efficient. Therefore, it has been widely used previously to characterize secreted proteins (Takken et al. 2000). The eukaryotic expression system has the ability to perform post-translational modifications, making the combined protein closer to the original structure of the native protein. Therefore, it is also used in some studies on protein functional characterization. The

prokaryotic expression system has high expression efficiency. Although some studies have verified its feasibility in characterizing CDIP, the frequent formation of inclusion bodies and non-uniform expression conditions limit its application in large-scale CDIP screening (Zhang et al. 2017; Zhu et al. 2017). It has been suggested that excessive and rapid expression and incorrect disulfide bond pairing are the main reasons for forming inclusion bodies (Paraskevopoulou and Falcone 2018). Several strategies have been proposed in recent years to solve the formation of inclusion bodies, such as optimizing strains, lowering the temperature, and co-expressing molecular chaperones (Rosano and Ceccarelli 2014). Since most of the secreted proteins have small molecular weights, they can be synthesized rapidly in *E. coli* and are prone to form inclusion bodies. Here, we explored different expression conditions to optimize the prokaryotic expression system for screening CDIP. Co-expression of CDIP with the molecular chaperone TF at 15 °C efficiently solved the CDIP inclusion body formation problem (Fig. S1). TF is derived from *E. coli* with outstanding solubility and has been proposed as a folding catalyst, effectively preventing the aggregation of recombinant proteins (Nishihara et al. 2000). Furthermore, we verified that high concentrations of TF have no obvious impact on plants, which suggests that the optimized prokaryotic expression system is suitable for CDIP screening (Fig. 1).

To determine the expression system for large-scale CDIP screening, we further compared the effectiveness of different expression systems for validating reported CDIPs in *B. cinerea*. The results showed that the previously reported BcSPL1 expressed by the transient expression system failed to induce cell death, possibly because the transiently expressed protein level was insufficient. Therefore, the *Agrobacterium*-mediated transient expression system may miss some CDIPs during large-scale screening. Although the eukaryotic expression system showed excellent screening effectiveness, it is unsuitable for large-scale screening due to its time-consuming and labor-intensive properties (Fig. 2). Consequently, we chose the prokaryotic expression system as the final screening system.

Through the prokaryotic expression system, we validated the cell death-inducing activity of 55 proteins, mainly cell wall-degrading enzymes, immune-associated proteins, proteases, and oxidoreductases. Among the 55 candidate proteins, two SGNH family CDIPs, BcRAE and BcFAT, were identified. The SGNH hydrolase superfamily (once called the GDSL family), named after the four conserved residues serine, glycine, asparagine, and histidine, is widely found in prokaryotes and eukaryotes with a broad range of substrates (Lescic Asler et al. 2017). The SGNH hydrolase superfamily comprises a diverse range

of carbohydrate-modifying enzymes, including but not limited to carbohydrate esterase (Anderson et al. 2022). The crystal structures of SGNH family proteins have been extensively studied (Mlgaard et al. 2000; Lescic Asler et al. 2017; Anderson et al. 2022). SGNH hydrolases adopt an alpha/beta/alpha-sandwich fold. Enzymes containing this domain act as esterases and lipases but have little sequence homology to true lipases. Furthermore, our results showed that *BcRAE* and *BcFAT* were highly expressed during infection, suggesting a potential role of *BcRAE* and *BcFAT* in *B. cinerea*-plant interactions (Fig. 5). Notably, signal peptide prediction indicates that *BcFAT* lacks a signal peptide. Nevertheless, *BcFAT* has been identified in multiple secretome studies (Shah et al. 2009a, 2009b; Espino et al. 2010; Zhu et al. 2017; Liu et al. 2023), suggesting that *BcFAT* may be secreted to the extracellular space through an unconventional secretion pathway. Besides the classical protein secretion pathway mediated by signal peptides, several unconventional protein secretion routes have been reported in eukaryotes (Zhang and Schekman 2013; Rabouille 2017). *BcGS1*, a previously reported CDIP without a signal peptide, was identified in the secreted proteins of *B. cinerea* (Zhang et al. 2015). Additionally, several virulence factors in other fungi have been reported to be secreted to the extracellular space through non-classical secretion pathways (Artier et al. 2018; Li et al. 2019). Previous studies have reported that SGNH hydrolases in plants play different roles in plant immunity. *Arabidopsis* GDSL-like lipase 1 (*GLIP1*) has been reported to play an essential role in plant immunity by regulating local and systemic resistance in plants through the ethylene pathway. Overexpression of *GLIP1* in *Arabidopsis* enhanced plant resistance, such as *Alternaria brassicicola*, *Erwinia carotovora*, and *Pseudomonas syringae* (Oh et al. 2005; Kwon et al. 2009; Kim et al. 2013). However, silencing *CaGLIP1* in pepper increased resistance to *Xanthomonas campestris*, and overexpression of *CaGLIP1* in *Arabidopsis* reduced plant resistance to *P. syringae* and *Hyaloperonospora parasitica* (Hong et al. 2008). Moreover, *OsGLIP1* and *OsGLIP2* were reported to negatively regulate rice resistance to *Xanthomonas oryzae* and *Magnaporthe oryzae* by modulating lipid metabolism (Gao et al. 2017).

It has been reported that the necrosis-inducing activity of some CDIPs is dose-dependent (Frias et al. 2016; Zhu et al. 2022). Our results show that *BcFAT* and *BcRAE* at 50  $\mu$ M triggered intense cell death. Symptoms were weakened with decreasing protein concentrations (Fig. 4). When the protein concentration was as low as 1  $\mu$ M, both failed to trigger necrosis, suggesting that the necrosis-inducing activity of *BcFAT* and *BcRAE* is dose-dependent, which also explains the inability of transiently expressed *BcFAT* and *BcRAE* to trigger

necrotic symptoms. Some CDIPs have been reported to induce cell death independent of their enzymatic activities. For example, *Bcxyn11* triggers necrosis in tobacco, and its necrosis-inducing activity does not depend on its xylanase activity (Noda et al. 2010). Similar results for *BcXYG1* and *BcCRH1* were reported (Zhu et al. 2017; Bi et al. 2021). We also examined whether the cell death-inducing activities of *BcFAT* and *BcRAE* depended on their SGNH hydrolase activities. The results showed that the cell death-inducing activities of both *BcFAT* and *BcRAE* were abolished when the conserved catalytic residue Ser was mutated to Ala. In addition, high-temperature denaturation also abolished the cell death-inducing activities of both *BcFAT* and *BcRAE*. These results suggest that enzyme activities are indispensable for *BcRAE* and *BcFAT*-induced cell death. Since both *BcRAE* and *BcFAT* target cell wall components, the degradation products of the enzymes may function as damage-associated molecular patterns (DAMPs) to trigger cell death in plants. Similar results have been reported before. For example, It has been reported that *Mocel12A/B* of *M. oryzae* does not trigger the immune response by itself, but oligosaccharides derived from the degradation of the cell wall by *Mocel12A/B* activate the immune response in plants (Yang et al. 2021). The effect of *BcFAT* and *BcRAE* degradation products on plant immunity needs further study. Moreover, our results show that the single and double knockout of *BcFAT* and *BcRAE* did not affect the growth and virulence of *B. cinerea* (Fig. S3). This result is unsurprising because there are four homologous proteins of *BcFAT* and two homologous proteins of *BcRAE* in *B. cinerea*. We speculated that the functional redundancy among homologous proteins or CDIPs might lead to the absence of a significant phenotype in these mutant strains. Similar results have been reported for *BcXYG1*, *BcCRH1*, and *BcIEB1* (Frias et al. 2016; Zhu et al. 2017; Bi et al. 2021).

During long-term co-evolution with pathogens, plants have developed a sophisticated immune system to resist the attack of pathogens. Conserved pathogen-associated molecular patterns (PAMPs) from pathogens and DAMPs from plants are recognized by plant pattern recognition receptors (PRRs) embedded in the plasma membrane and activate pattern-triggered immunity (PTI). Specific adapted pathogens further secrete effectors to suppress PTI, while intracellular disease resistance proteins specifically recognize effectors and activate effector-triggered immunity (ETI) (Xu et al. 2022). Here, we found that *BcFAT* and *BcRAE* are highly expressed during early infection. Abundantly expressed *BcFAT* and *BcRAE* are sufficient to induce cell death, which depends on their enzymatic activities. Nevertheless, transient expression of *BcRAE* and *BcFAT* could not induce cell death but

enhanced plant resistance to *B. cinerea*. Moreover, transient expression of site-mutated BcRAE and BcFAT could still induce plant resistance against *B. cinerea*. These results indicate that the resistance-inducing activities of BcFAT and BcRAE are independent of their enzymatic activities, which differs from their cell death-inducing activities. We speculate that BcFAT and BcRAE play a two-faced role during the plant-*B. cinerea* interaction. During early infection, *B. cinerea* secretes abundant BcFAT and BcRAE to kill plant cells by destroying cell walls. As a response, plants evolve corresponding receptors to recognize them and activate plant immunity to limit the infection of *B. cinerea*. Therefore, exploring BcRAE and BcFAT recognition proteins in plants would be interesting. Analysis of immune-related gene expression showed that BcRAE induces the expression of *NbPR1b* but suppresses *NbPR1a*. *PR1* is commonly considered a marker of systemic acquired resistance (Vleeshouwers et al. 2000; Boyle et al. 2009; Frackowiak et al. 2019). Unlike BcRAE, BcFAT had no significant effect on *NbPR1a/b* expression, suggesting that BcFAT may act through a different pathway. Both BcFAT and BcRAE resulted in the upregulation of *NbPTIS* and *NbrBOHb*. *PTIS* is a marker of the PTI response and regulates a series of defense-related genes downstream (Nguyen et al. 2010). The activation of *NbrBOHb* is associated with a reactive oxygen species (ROS) burst, an early event of the plant immune response (Irieda et al. 2018). The qRT-PCR results are consistent with enhanced resistance in plants. These results suggest that BcRAE and BcFAT may trigger the plant PTI response and induce the expression of downstream defense genes and the burst of ROS, thus enhancing resistance to *B. cinerea*.

In conclusion, we compared different protein expression systems and suggested that the optimized prokaryotic expression system is suitable for high-throughput CDIP screening. With the help of the prokaryotic expression system, two novel SGNH family CDIPs, BcRAE and BcFAT, were identified. We also preliminarily resolved the mechanism of BcFAT and BcRAE. Our results provide new targets for further study of *B. cinerea*-plant interactions and lay a theoretical foundation for controlling *B. cinerea*.

## Methods

### Fungal strains and plant materials

*Botrytis cinerea* (B05.10) was used as the wild-type and recipient strain to generate mutant strains. All strains were cultured in continuous light on potato dextrose agar (PDA) medium at 22 °C. *Nicotiana benthamiana* was cultivated in a culture room at 23 °C with 16 h of daily illumination.

### Candidate-secreted protein selection and plasmid construction

Candidate-secreted proteins were selected from previously reported secretomes of *B. cinerea* (Shah et al. 2009a; Shah et al. 2009b; Espino et al. 2010; Li et al. 2012; Zhu et al. 2017; Li et al. 2020; Liu et al. 2023). The candidate gene sequences were obtained from NCBI (<http://www.ncbi.nlm.nih.gov>) and EnsemblFungi ([http://fungi.ensembl.org/Botrytis\\_cinerea/Info/Index](http://fungi.ensembl.org/Botrytis_cinerea/Info/Index)). The protein function and signal peptide information were retrieved from EnsemblFungi.

Total RNA was extracted from *B. cinerea* mycelia using TRNzol reagent (Tiangen, China) as previously described (Zhang et al. 2021b). cDNA was synthesized using the PrimeScript RT reagent Kit (Takara, Japan) following the manufacturer's instructions. The ORF sequences of target genes without signal peptides were amplified from the synthesized cDNA. The linearized vectors pPIC9K and pCold-TF were used to construct eukaryotic and prokaryotic expression vectors, respectively. For the construction of *Agrobacterium*-mediated transient expression vectors, the *Arabidopsis* *PR3* signal peptide was fused to the N-terminus of the PVX-3×HA vector according to the description of Zhu et al. (2017) to form PVX-SP-3×HA vector that enables proteins to be secreted to the apoplast. The PVX-SP-3×HA vector was linearized with the restriction enzymes ClaI and NotI. The amplified fragments were fused with the linearized expression vectors by homologous recombination using an SE Seamless Cloning and Assembly Kit (Zoman, China). All constructed vectors were confirmed by sequencing. The primers used in this paper are listed in Supplementary Table S1.

### Protein expression and cell death-induction assay

Protein expression in the eukaryotic system was conducted following the protocol by Zhang et al. (2017) with minor adjustments. In brief, the pPIC9K vector fused with the candidate gene was linearized by Sall and then transformed into *Pichia pastoris* GS115, and an empty pPIC9K vector was introduced as the control. A Mut<sup>+</sup> (methanol utilization plus phenotype) transformant carrying multi-copy expression cassettes was screened by methanol and geneticin (G418) for subsequent protein expression. The transformant was cultured in BMMY medium with 0.5% methanol for 48 h. The crude protein was precipitated from the supernatant of the liquid culture by saturated ammonium sulfate solution. The 6×His labeled recombinant protein was then purified using Ni-NTA agarose (Qiagen, USA) and verified by gel electrophoresis. Finally, with the help of Amicon Ultra Centrifugal Filter Units (Merck Millipore, Germany), verified proteins were concentrated, and the buffer was

exchanged with PBS buffer (0.01 M phosphate buffer solution, pH 7.4).

The prokaryotic expression of proteins was conducted following Xing et al. (2021), with slight modifications. Constructed pET30a or pCold-TF plasmids were introduced into *Escherichia coli* strain *Rosetta*. Positive colonies were incubated in 10 mL ampicillin-supplemented LB broth medium at 37 °C overnight. The overnight-incubated *E. coli* was added to 1 L of fresh LB broth containing ampicillin and cultured at 37 °C until the OD<sub>600</sub> reached 0.5. IPTG induced the culture with a final concentration of 0.5 mM. For expression with the pET30a vector, the culture was induced at 37 °C for 5 h or 15 °C for 16 h. For expression with the pCold-TF vector, the culture was incubated at 15 °C for 16 h. *E. coli* cells were collected, resuspended, and sonicated on ice until the solution was clear. The lysate was centrifuged at 8000 g at 4 °C for 20 min. The supernatant and precipitate were collected separately for SDS-PAGE. Protein purification, verification, concentration, and buffer replacement were the same as described in the eukaryotic expression system. Protein concentrations were measured by Bradford assay with a BSA standard curve (Bradford 1976).

*Agrobacterium*-mediated transient expression was performed according to Wang et al. (2011). The recombinant plasmids were introduced into *Agrobacterium tumefaciens* strain GV3101 (pJIC SA\_Rep) by electroporation, and the empty PVX-SP-3×HA vector was transformed as the control. The positive transformants were cultured in LB broth with kanamycin (50 mg L<sup>-1</sup>) and rifampicin (50 mg L<sup>-1</sup>). Cultured *Agrobacterium* cells were collected, washed, and resuspended in 10 mM MgCl<sub>2</sub>. The OD<sub>600</sub> was finally adjusted to 0.5. The suspension was then incubated at room temperature for 1 h before infiltration.

For the cell death induction assay, the *Agrobacterium* cell suspension, 50 μM purified prokaryotically expressed protein, and 50 μM purified eukaryotically expressed protein were infiltrated into plant leaves. For high-temperature denaturation, proteins were incubated at 95 °C for 15 min. Each assay contained at least nine leaves. Cell death symptoms were monitored and recorded 3 to 7 d after infiltration.

#### Gene expression pattern analysis

For the gene expression pattern analysis, *B. cinerea* conidia (2×10<sup>5</sup> spores mL<sup>-1</sup>) were inoculated on tomato fruits or into PDB medium. Samples were collected at 6, 12, 24, and 48 hpi, and RNA extraction was subsequently performed. The qRT-PCR was utilized to detect the relative expression levels of genes (Liu et al. 2023). *BctubA* (BCIN\_01g08040) served as the reference gene, and the gene expression level of *B. cinerea* cultured in PDB for 6 h was set to 1.

#### Site-directed mutagenesis

The process of site-directed mutagenesis was carried out using the M5 HiPer Site-Directed Mutagenesis Kit (Mei5bio, China) following the manufacturer's instructions. The pCold-TF plasmids harboring the ORF of *BcFAT* or *BcRAE* were used as templates to obtain site-directed mutants. The primer pairs for mutagenesis were designed using an online design tool (<http://ps.biocloud.org.cn/>). Site-mutated gene fragments were amplified from mutated pCold vectors and then homologously recombined into the PVX vector to construct transient expression vectors of site-mutated proteins.

#### Generation of knockout mutants and phenotype assay

The knockout mutant generation and phenotype assay followed a previously described procedure (Li et al. 2020). The gene knockout of *B. cinerea* was based on the gene replacement strategy. In brief, the hygromycin resistance cassette flanked by the upstream and downstream regions of *BcFAT* or *BcRAE* was transformed into protoplasts of wild-type *B. cinerea*. Hygromycin B was used to select positive transformants, followed by single-spore isolation to isolate homokaryons. The homokaryons were verified by flank-spanning PCR. The  $\Delta BcFAT/BcRAE$  double knockout mutant was generated based on the  $\Delta BcRAE$  single knockout mutant, according to Zhang et al. (2021b). The nourseothricin resistance cassette was amplified from the pNAN-OGG vector and fused with the upstream and downstream regions of *BcFAT*. The fused fragment was transformed into protoplasts of the  $\Delta BcRAE$  mutant strain, and positive transformants were screened on PDA plates containing nourseothricin.

For growth phenotype assay, the homokaryotic mutants and wild-type were cultured on PDA medium plates at 22 °C under light. The colony diameters of cultures were measured 2 to 3 days postinoculation. The assay was repeated three times. For the pathogenic phenotype assay, spores of wild-type and mutants were inoculated on sterilized apple fruits (*Malus pumila* Mill cv. Fuji). Lesion development was monitored and recorded three days postinoculation. At least nine apples were used in each assay. The experiment contained three repeats.

#### Induction of plant resistance and defense-related gene expression

To investigate the effect of *BcFAT* and *BcRAE* on plant resistance, *BcFAT* and *BcRAE* were expressed in *N. benthamiana* through the *Agrobacterium*-mediated transient expression system described above. Protein-expressing *Agrobacterium* and the control *Agrobacterium* infiltrated different sides of the same leaf. The treated tobacco was kept in the culture room for three days, followed by inoculation of 10 μL of *B. cinerea* spore suspension (2×10<sup>5</sup> spores mL<sup>-1</sup>)

diluted PDB) on the *Agrobacterium*-infiltrated site. WB was performed to detect the expression of proteins. The inoculated plants were kept in a humid chamber at 22 °C under the light. The lesion development was recorded 60 h post-inoculation. Each assay contained at least nine leaves, and the experiment was repeated three times. *N. benthamiana* leaves were infiltrated with 50 μM control protein, BcFAT, and BcRAE to detect the defense-related gene expression. After 24 h of growth in the culture room, the treated leaves were picked for RNA extraction and qRT-PCR analysis. The experiment was repeated three times.

### Statistical analysis

Statistical differences in phenotype assay, resistance induction, and defense-related gene expression experiments were analyzed using IBM SPSS Statistics 21 software (IBM, USA). Comparisons were performed through one-way ANOVA with Tukey's test, and a *P*-value < 0.05 was considered significant.

### Abbreviations

CDIP	Cell death-inducing protein
DAMP	Damage-associated molecular pattern
ETI	Effector-triggered immunity
GLIP	GDSL-like lipase
hpi	Hours post-inoculation
PAMP	Pathogen-associated molecular pattern
PTI	Pattern-triggered immunity
ROS	Reactive oxygen species
SD	Standard deviation
TF	Trigger factor
WB	Western blot

### Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s43897-024-00086-3>.

**Additional file 1: Table S1.** Information on candidate proteins and primers used in this paper.

**Additional file 2: Figure S1.** Optimization of prokaryotic expression conditions.

**Additional file 3: Figure S2.** Expression of candidate proteins by the prokaryotic expression system.

**Additional file 4: Figure S3.** Phenotypes of BcFAT and BcRAE knockout mutant strains.

### Acknowledgements

We thank Prof. Yuanchao Wang (Nanjing Agricultural University) for kindly providing the PVX-3xHA vector.

### Authors' contributions

XZ performed the experiments and analyzed the data. XZ and ZZ wrote the original draft. TC, YC, and BL offered professional assistance in analyzing data and revising the manuscript. ST designed the research and revised the manuscript. All authors have read and approved the final manuscript.

### Funding

This study was supported by the National Natural Science Foundation of China (31930086, U23A20264, 31972475) and the National Key R&D Program of China (2021YFD2100505).

### Availability of data and materials

The data will be available from the corresponding author upon reasonable request.

### Declarations

#### Ethics approval and consent to participate

Not applicable.

#### Consent for publication

Not applicable.

#### Competing interests

The authors declare that they have no competing interests. Prof. Shi-Ping Tian is a member of the Editorial Board for *Molecular Horticulture*. She was not involved in the journal's review of, and decisions related to, this manuscript.

Received: 20 November 2023 Accepted: 7 February 2024

Published online: 07 March 2024

### References

- Akoh CC, Lee GC, Liaw YC, Huang TH, Shaw JF. GDSL family of serine esterases/lipases. *Prog Lipid Res.* 2004;43(6):534–52.
- An B, Li BQ, Li H, Zhang ZQ, Qin GZ, Tian SP. Aquaporin8 regulates cellular development and reactive oxygen species production, a critical component of virulence in *Botrytis cinerea*. *New Phytol.* 2015;209(4):1668–80.
- Anderson AC, Stangherlin S, Pimentel KN, Weadge JT, Clarke AJ. The SGNH hydrolase family: a template for carbohydrate diversity. *Glycobiology.* 2022;32(10):826–48.
- Artier J, da Silva Zandonadi F, de Souza Carvalho FM, Pauletti BA, Leme AFP, Carnielli CM, et al. Comparative proteomic analysis of *Xanthomonas citri* ssp. *citri* periplasmic proteins reveals changes in cellular envelope metabolism during in vitro pathogenicity induction. *Mol Plant Pathol.* 2018;19(1):143–57.
- Bi K, Scalschi L, Jaiswal N, Mengiste T, Fried R, Sanz AB, et al. The *Botrytis cinerea* Crh1 transglycosylase is a cytoplasmic effector triggering plant cell death and defense response. *Nat Commun.* 2021;12(1):2166.
- Bi K, Liang Y, Mengiste T, Sharon A. Killing softly: a roadmap of *Botrytis cinerea* pathogenicity. *Trends Plant Sci.* 2023;28(2):211–22.
- Boyle P, Le Su E, Rochon A, Shearer HL, Murmu J, Chu JY, et al. The BTB/POZ domain of the *Arabidopsis* disease resistance protein NPR1 interacts with the repression domain of TGA2 to negate its function. *Plant Cell.* 2009;21(11):3700–13.
- Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem.* 1976;72(1):248–54.
- Chen DG, Chen T, Chen Y, Zhang ZQ, Li B, Tian SP. Bio-source substances against postharvest diseases of fruits: Mechanisms, applications and perspectives. *Postharvest Biol Technol.* 2023a;198:112240.
- Chen T, Zhang ZQ, Chen Y, Li BQ, Tian SP. *Botrytis cinerea*. *Curr Biol.* 2023b;33(11):R460–2.
- Cui XM, Ma DY, Liu XY, Zhang ZQ, Li BQ, Xu Y, et al. Magnolol inhibits gray mold on postharvest fruit by inducing autophagic activity of *Botrytis cinerea*. *Postharvest Biol Technol.* 2021;180:111596.
- Dean R, van Kan JAL, Pretorius ZA, Hammond-Kosack KE, Di Pietro A, Spanu PD, et al. The Top 10 fungal pathogens in molecular plant pathology. *Mol Plant Pathol.* 2012;13(4):414–30.
- Espino JJ, Gutiérrez-Sánchez G, Brito N, Shah P, Orlando R, González C. The *Botrytis cinerea* early secretome. *Proteomics.* 2010;10(16):3020–34.
- Frackowiak P, Pospieszny H, Smiglak M, Obrepalska-Stepłowska A. Assessment of the efficacy and mode of action of Benzo(1,2,3)-Thiadiazole-7-Carbothioic Acid S-Methyl Ester (BTH) and its derivatives in plant protection against viral disease. *Int J Mol Sci.* 2019;20(7):1589.
- Frías M, González C, Brito N. BcSpl1, a cerato-platanin family protein, contributes to *Botrytis cinerea* virulence and elicits the hypersensitive response in the host. *New Phytol.* 2011;192(2):483–95.

- Frías M, González M, González C, Brito N. BclEB1, a *Botrytis cinerea* secreted protein, elicits a defense response in plants. *Plant Sci.* 2016;250:115–24.
- Gao MJ, Yin X, Yang WB, Lam SM, Tong XH, Liu JY, et al. GDSL lipases modulate immunity through lipid homeostasis in rice. *PLoS Pathog.* 2017;13(11):e1006724.
- González M, Brito N, González C. The *Botrytis cinerea* elicitor protein BclEB1 interacts with the tobacco PR5-family protein osmotin and protects the fungus against its antifungal activity. *New Phytol.* 2017;215(1):397–410.
- Gu N, Zhang XY, Gu XY, Zhao LN, Godana EA, Xu MQ, et al. Transcriptomic and proteomic analysis of the mechanisms involved in enhanced disease resistance of strawberries induced by *Rhodotorula mucilaginosa* cultured with chitosan. *Postharvest Biol Technol.* 2021;172:111355.
- Hong JK, Choi HW, Hwang IS, Kim DS, Kim NH, Choi DS, et al. Function of a novel GDSL-type pepper lipase gene, CaGLIP1, in disease susceptibility and abiotic stress tolerance. *Planta.* 2008;227(3):539–58.
- Irieda H, Inoue Y, Mori M, Yamada K, Oshikawa Y, Saitoh H, et al. Conserved fungal effector suppresses PAMP-triggered immunity by targeting plant immune kinases. *P Natl Acad Sci USA.* 2018;116(2):496–505.
- Jeblick T, Leisen T, Steidele CE, Albert I, Muller J, Kaiser S, et al. *Botrytis* hypersensitive response inducing protein 1 triggers noncanonical PTI to induce plant cell death. *Plant Physiol.* 2023;191(1):125–41.
- Kabbage M, Williams B, Dickman MB. Cell death control: the interplay of apoptosis and autophagy in the pathogenicity of *Sclerotinia sclerotiorum*. *PLoS Pathog.* 2013;9(4):e1003287.
- Kim HG, Kwon SJ, Jang YJ, Nam MH, Chung JH, Na YC, et al. GDSL LIPASE1 modulates plant immunity through feedback regulation of ethylene signaling. *Plant Physiol.* 2013;163(4):1776–91.
- Kwon SJ, Jin HC, Lee S, Nam MH, Chung JH, Kwon SI, et al. GDSL lipase-like 1 regulates systemic resistance associated with ethylene signaling in *Arabidopsis*. *Plant J.* 2009;58(2):235–45.
- Lee DS, Kim BK, Kwon SJ, Jin HC, Park OK. *Arabidopsis* GDSL lipase 2 plays a role in pathogen defense via negative regulation of auxin signaling. *Biochem Biophys Res Commun.* 2009;379(4):1038–42.
- Leisen T, Werner J, Pattar P, Safari N, Ymeri E, Sommer F, et al. Multiple knockout mutants reveal a high redundancy of phytotoxic compounds contributing to necrotrophic pathogenesis of *Botrytis cinerea*. *PLoS Pathog.* 2022;18(3):e1010367.
- Lescic Asler I, Stefanic Z, Marsavelski A, Vianello R, Kojic-Prodic B. Catalytic dyad in the SGNH hydrolase superfamily: in-depth insight into structural parameters tuning the catalytic process of extracellular lipase from *Streptomyces rimosus*. *ACS Chem Biol.* 2017;12(7):1928–36.
- Li BQ, Wang WH, Zong YY, Qin GZ, Tian SP. Exploring pathogenic mechanisms of *Botrytis cinerea* secretome under different ambient pH based on comparative proteomic analysis. *J Proteome Res.* 2012;11(8):4249–60.
- Li GJ, Chen Y, Zhang ZQ, Li BQ, Chen T, Tian SP. 2,3-Butanedione suppresses gray mold of postharvest fruit by activating the autophagy of *Botrytis cinerea*. *Postharvest Biol Technol.* 2022a;193:112057.
- Li GJ, Wang Y, Zhang ZQ, Chen Y, Tian SP. Mushroom alcohol controls gray mold caused by *Botrytis cinerea* in harvested fruit via activating the genes involved in jasmonic acid signaling pathway. *Postharvest Biol Technol.* 2022b;186:111843.
- Li TT, Wu Y, Wang Y, Gao HY, Gupta VK, Duan XW, et al. Secretome profiling reveals virulence-associated proteins of *Fusarium proliferatum* during interaction with banana fruit. *Biomolecules.* 2019;9(6):246.
- Li H, Zhang ZQ, Qin GZ, He C, Li BQ, Tian SP. Actin is required for cellular development and virulence of *Botrytis cinerea* via the mediation of secretory proteins. *mSystems.* 2020;5(1):e00732–19.
- Liu K, Liu W, Huang XH, Liu YH, Cui XM, Zhang ZQ, et al. Identification of virulence-related proteins during *Botrytis cinerea* – fruit interaction at early phase. *Postharvest Biol Technol.* 2023;204:112443.
- Lu SS, Zhang XK, He C, Li GJ, Chen T, Li BQ, et al. The Zn(II)<sub>2</sub>Cys<sub>6</sub> transcription factor BcDIC affects the asexual reproduction of *Botrytis cinerea* by regulating pectinesterase genes. *Phytopathol Res.* 2023;5(1):35.
- Mlgaard A, Kauppinen S, Larsen S. Rhamnolacturonan acetyltransferase elucidates the structure and function of a new family of hydrolases. *Structure.* 2000;8(4):373–83.
- Nguyen HP, Chakravarthy S, Velásquez A, McLane HL, Martin GB. Methods to study PAMP-triggered immunity using tomato and *Nicotiana benthamiana*. *Mol Plant Microbe in.* 2010;23(8):991–9.
- Nishihara K, Kanemori M, Yanagi H, Yura T. overexpression of trigger factor prevents aggregation of recombinant proteins in escherichia coli. *Appl Environ Microbiol.* 2000;66(3):884–9.
- Noda J, Brito N, Gonzalez C. The *Botrytis cinerea* xylanase Xyn11A contributes to virulence with its necrotizing activity, not with its catalytic activity. *BMC Plant Biol.* 2010;10:38.
- Oh IS, Park AR, Bae MS, Kwon SJ, Kim YS, Lee JE, et al. Secretome analysis reveals an *Arabidopsis* lipase involved in defense against *Alternaria brassicicola*. *Plant Cell.* 2005;17(10):2832–47.
- Paraskevopoulou V, Falcone F. Polyionic tags as enhancers of protein solubility in recombinant protein expression. *Microorganisms.* 2018;6(2):47.
- Poveda J, Barquero M, González-Andrés F. Insight into the microbiological control strategies against *Botrytis cinerea* using systemic plant resistance activation. *Agronomy.* 2020;10(11):1822.
- Rabouille C. Pathways of unconventional protein secretion. *Trends Cell Biol.* 2017;27(3):230–40.
- Rosano GL, Ceccarelli EA. Recombinant protein expression in *Escherichia coli*: advances and challenges. *Front Microbiol.* 2014;5:172.
- Shah P, Atwood JA, Orlando R, Mubarek HE, Davis MR. Comparative proteomic analysis of *Botrytis cinerea* secretome. *J Proteome Res.* 2009a;8(3):1123–30.
- Shah P, Gutierrez-Sanchez G, Orlando R, Bergmann C. A proteomic study of pectin-degrading enzymes secreted by *Botrytis cinerea* grown in liquid culture. *Proteomics.* 2009b;9(11):3126–35.
- Shlezinger N, Minz A, Gur Y, Hatam I, Dagdas YF, Talbot NJ, et al. Anti-apoptotic machinery protects the necrotrophic fungus *Botrytis cinerea* from host-induced apoptotic-like cell death during plant infection. *PLoS Pathog.* 2011;7(8):e1002185.
- Sun YY, Wang Y, Xu Y, Chen T, Li BQ, Zhang ZQ, et al. Application and mechanism of benzyl-isothiocyanate, a natural antimicrobial agent from cruciferous vegetables, in controlling postharvest decay of strawberry. *Postharvest Biol Technol.* 2021;180:111604.
- Takken FL, Luderer R, Gabriels SH, Westerink N, Lu R, de Wit PJ, et al. A functional cloning strategy, based on a binary PVX-expression vector, to isolate HR-inducing cDNAs of plant pathogens. *Plant J.* 2000;24(2):275–83.
- Veloso J, van Kan JAL. Many shades of grey in *Botrytis*-host plant interactions. *Trends Plant Sci.* 2018;23(7):613–22.
- Vleeshouwers VGAA, van Dooijeweert W, Govers F, Kamoun S, Colon LT. oes basal PR gene expression in *Solanum* species contribute to non-specific resistance to *Phytophthora infestans*? *Physiol Mol Plant P.* 2000;57(1):35–42.
- Wang QQ, Han CZ, Ferreira AO, Yu XL, Ye WW, Tripathy S, et al. Transcriptional programming and functional interactions within the *Phytophthora sojae* RXLR effector repertoire. *Plant Cell.* 2011;23(6):2064–86.
- Wang Y, Liu XY, Chen T, Xu Y, Tian SP. Antifungal effects of hinokitiol on development of *Botrytis cinerea* in vitro and in vivo. *Postharvest Biol Technol.* 2020;159:111038.
- Xing MY, Chen Y, Li BQ, Tian SP. Characterization of a short-chain dehydrogenase/reductase and its function in patulin biodegradation in apple juice. *Food Chem.* 2021;348:129046.
- Xu XD, Chen Y, Li BQ, Zhang ZQ, Qin GZ, Chen T, et al. Molecular mechanisms underlying multi-level defense responses of horticultural crops to fungal pathogens. *Hortic Res.* 2022;9:uhac066.
- Yang GG, Tang LG, Gong YD, Xie JT, Fu YP, Jiang DH, et al. A cerato-platanin protein SsCP1 targets plant PR1 and contributes to virulence of *Sclerotinia sclerotiorum*. *New Phytol.* 2018a;217(2):739–55.
- Yang YK, Yang XF, Dong YJ, Qiu DW. The *Botrytis cinerea* xylanase BcXyl1 modulates plant immunity. *Front Microbiol.* 2018b;9:2535.
- Yang C, Liu R, Pang JH, Ren B, Zhou HB, Wang G, et al. Poaceae-specific cell wall-derived oligosaccharides activate plant immunity via OsCERK1 during *Magnaporthe oryzae* infection in rice. *Nat Commun.* 2021;12(1):2178.
- Yang XM, Wang YT, Jiang HY, Song R, Liu YG, Guo HL, et al. Antimicrobial peptide CB-M exhibits direct antifungal activity against *Botrytis cinerea* and induces disease resistance to gray mold in cherry tomato fruit. *Postharvest Biol Technol.* 2023;196:112184.
- Yigal E, Ilaria P, Alba M, Cotes P, Alison S. Plant hosts of *Botrytis* spp. In: Fillingier S, Elad Y, editors. *Botrytis – the fungus, the pathogen and its management in agricultural systems*. Springer International Publishing; 2016. p. 413–86.
- Zhang M, Schekman R. Unconventional secretion, unconventional solutions. *Science.* 2013;340(6132):559–61.
- Zhang Y, Zhang YH, Qiu DW, Zeng HM, Guo LH, Yang XF. BcGs1, a glycoprotein from *Botrytis cinerea*, elicits defence response and improves disease resistance in host plants. *Biochem Biophys Res Commun.* 2015;457(4):627–34.

- Zhang Y, Liang YB, Qiu DW, Yuan JJ, Yang XF. Comparison of cerato-platanin family protein BcSpl1 produced in *Pichia pastoris* and *Escherichia coli*. *Protein Expr Purif*. 2017;136:20–6.
- Zhang ZQ, Chen T, Li BQ, Qin GZ, Tian SP. Molecular basis of pathogenesis of postharvest pathogenic fungi and control strategy in fruits: progress and prospect. *Mol Horticulture*. 2021a;1:2.
- Zhang ZQ, He C, Chen Y, Li BQ, Tian SP. DNA methyltransferases regulate pathogenicity of *Botrytis cinerea* to horticultural crops. *J Fungi*. 2021b;7(8):659.
- Zhang XK, Li GJ, Zhang ZQ, Tian SP. 3-Octanol controls gray mold on post-harvest fruit by inducing autophagy of *Botrytis cinerea*. *Postharvest Biol Technol*. 2023;205:112525.
- Zhu WJ, Ronen M, Gur Y, Minz-Dub A, Masrati G, Ben-Tal N, et al. BcXYG1, a secreted xyloglucanase from *Botrytis cinerea*, triggers both cell death and plant immune responses. *Plant Physiol*. 2017;175(1):438–56.
- Zhu WJ, Yu MX, Xu R, Bi K, Yu S, Xiong C, et al. *Botrytis cinerea* BcSSP2 protein is a late infection phase, cytotoxic effector. *Environ Microbiol*. 2022;24(8):3420–35.

### **Publisher's Note**

Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.