Received: 21 May 2024 | Revised: 27 September 2024 | Accepted: 15 October 2024

DOI: 10.1111/mpp.70023

Molecular Plant Pathology WILEY

ORIGINAL ARTICLE

A novel protein elicitor (Cs08297) from *Ciboria shiraiana* enhances plant disease resistance

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Funding information
Chongqing Talent Program, Grant/Award Number: cstc2024ycjh-bgzxm0086; Hainan Province Science and Technology Special Fund, Grant/Award Number: ZDYF2022SHFZ319; Earmarked Fund for China Agriculture Research System, Grant/Award Number: CARS-18-ZJ0201; Chongqing Modern Agricultural Industry Technology System, Grant/Award Number: CQMAITS202311

Abstract

Ciboria shiraiana is a necrotrophic fungus that causes mulberry sclerotinia disease resulting in huge economic losses in agriculture. During infection, the fungus uses immunity elicitors to induce plant tissue necrosis that could facilitate its colonization on plants. However, the key elicitors and immune mechanisms remain unclear in *C. shiraiana*. Herein, a novel elicitor Cs08297 secreted by *C. shiraiana* was identified, and it was found to target the apoplast in plants to induce cell death. Cs08297 is a cysteine-rich protein unique to *C. shiraiana*, and cysteine residues in Cs08297 were crucial for its ability to induce cell death. Cs08297 induced a series of defence responses in *Nicotiana benthamiana*, including the burst of reactive oxygen species (ROS), callose deposition, and activation of defence-related genes. Cs08297 induced-cell death was mediated by leucine-rich repeat (LRR) receptor-like kinases BAK1 and SOBIR1. Purified His-tagged Cs08297-thioredoxin fusion protein triggered cell death in different plants and enhanced plant resistance to diseases. *Cs08297* was necessary for sclerotial development, oxidative-stress adaptation, and cell wall integrity but negatively regulated virulence of *C. shiraiana*. In conclusion, our results revealed that Cs08297 is a novel fungal elicitor in fungi inducing plant immunity. Furthermore, its potential to enhance plant resistance provides a new target to control agricultural diseases biologically.

KEYWORDS
cell death, *Ciboria shiraiana*, disease resistance, elicitor, plant immunity

1 | INTRODUCTION

Mulberry belongs to the genus *Morus* in the *Moraceae* family. It is widely cultivated around the world, especially in Asia (Jiao et al., 2020). Mulberry fruit is loved for the taste, colour, low calorie content, high nutritional value, and benefit to human health (Huang et al., 2017; Jelled et al., 2017). There are multiple active compounds in mulberry with antibacterial and anticancer properties, as well as potential in preventing cardiovascular diseases (Chen et al., 2016; Peng et al., 2011; Yuan & Zhao, 2017). However, mulberry is faced with the attack of many pathogens. For example, mulberry sclerotinia disease is caused by the necrotrophic fungus *Ciboria shiraiana*, leading to substantial reduction in mulberry yield and causing substantial economic losses (Zhang et al., 2021b). At present, chemical pesticides are widely used to control the pathogens. The over-reliance on chemical agents may contribute to environmental

pollution, pathogen resistance, and other associated concerns. Hence, it is urgent to develop environmentally friendly and effective disease control methods.

Necrotrophic pathogens infecting plants can adopt aggressive and versatile virulence strategies to induce cell death and obtain nutrients conducive to growth and reproduction from dead cells (Mengiste, 2012). Among these strategies, pathogens secrete various effectors to manipulate the plant immune response during infection (Cai et al., 2023). Fungal effectors can be classified into two primary categories: apoplastic effectors acting in extracellular spaces and cytoplasmic effectors functioning inside host cells (Giraldo et al., 2013). In response to invasive pathogens, plants have developed sophisticated immune systems for self-defence (Chang et al., 2022). The first layer of the immune system is activated by pathogen-associated molecular patterns (PAMPs) through the recognition by pattern recognition receptors (PRRs) (Macho & Zipfel, 2014). The response to PAMPs is termed PAMP-triggered immunity (PTI) (Bigeard et al., 2015). The second layer of the immune system is dependent on the plant's intracellular immune receptors, known as nucleotide-binding and leucine-rich repeat receptors (NLRs), which specifically recognize cytoplasmic effectors secreted by pathogens (Jones et al., 2016). After detection by NLRs, defences are induced; the process is referred to as effector-triggered immunity (ETI) (Ngou et al., 2022). These immune responses constitute integral components of the plant immune signal cascade, including calcium flux across the plasma membrane, dynamic changes in hormone levels, activation of MAPK protein kinase pathways, bursts of reactive oxygen species (ROS), callose deposition, and the expression of defence-related genes (Yuan et al., 2021).

The plant apoplast is a critical ecological battleground besieged by microbial pathogens, where the interplay between plants and microbes takes place (Du et al., 2016; Wang et al., 2020). During infection, pathogens deploy effectors to the plant apoplast, disrupting the physiological activities in hosts and thereby promoting infection. The biotrophic fungal pathogen *Cladosporium fulvum* secretes the effector protein Avr4, which mediates the activation of a defence cascade, ultimately leading to a hypersensitive response (HR) (Joosten et al., 1997). A small cysteine-rich protein VmE02 from *Valsa mali* is prevalent in oomycetes and fungi, exerting its impact by targeting the apoplast to induce cell death (Nie et al., 2019). *Phytophthora sojae* induces cell death by secreting the apoplastic effector PcEXLX1, eliciting a robust defence response in host plants (Pi et al., 2022). Through a growing amount of research, it has been shown that pathogens secrete apoplastic effectors to evoke plant immune responses. On the other hand, host plants recognize apoplastic effectors through plasma membrane-localized PRRs, and then basal immunity is triggered. Receptor-like proteins (RLPs) and receptor-like kinases (RLKs) have been identified as PRRs, and both possess a ligand-binding ectodomain (Monaghan & Zipfel, 2012). After recognizing specific ligands, LRR-type PRRs can form a complex with the coreceptor BRASSINOSTEROID INSENSITIVE 1-associated receptor kinase 1 (BAK1)/SOMATIC EMBRYOGENESIS RECEPTOR KINASE 3 (SERK3). RLK-type PRRs, such as flagellin-sensing 2, can associate with BAK1 and activate immune responses (Perraki et al., 2018). The plant immune response induced by Cs02526 of *C. shiraiana* is dependent on BAK1 (Zhang et al., 2024). RLPs share similarities with RLKs but the intracellular kinase domain is lacking. RLP-type PRRs frequently form constitutive associations with an adaptor LRR-RLK, namely, Suppressor of BIR1 (SOBIR1) (Gust & Felix, 2014). For example, the RLP32-SOBIR1-BAK1 complex recognizes necrosis and ethylene-inducing peptide 1-like proteins (NLPs), initiating a robust immune response in plants (Albert et al., 2015). Recent research showed that BAK1 and SOBIR1 directly phosphorylate each other and that residues Thr522 and Tyr469 of the kinase domain of *Nicotiana benthamiana* SOBIR1 are required for its kinase activity and for interacting with signalling partners, respectively (Huang et al., 2024). The sensing and signalling network of these complexes makes a significant contribution to the rapid defence of plants against pathogens and enhanced disease resistance.

Elicitors have emerged as a promising alternative to conventional fungicides and insecticides in sustainable agriculture. Biological control methods could diminish reliance on chemical fertilizers and pesticides, thereby minimizing environmental impact (Abdul Malik et al., 2020). In previous study, a novel protein elicitor named PeBL1, derived from *Brevibacillus laterosporus* A60, was identified. Treatment with PeBL1 induces a spectrum of defence responses in *N. benthamiana*, including an ROS burst, activation of defence-related genes, and accumulation of defence-related proteins (Wang et al., 2015). The *Sclerotinia sclerotiorum* protein elicitor SCFE1 induces typical PTI responses in *Arabidopsis thaliana* (Zhang et al., 2013). The protein elicitor PeSy1 of *Saccharothrix yanglingensis* induces a strong HR and resistance in plants (Wang et al., 2023). While function of elicitors in various pathogens has been studied, those in *C. shiraiana* have not been reported. The family *Sclerotiniaceae* is in the phylum Ascomycota, and includes species that cause Sclerotinia diseases in many economically important crops and forests. *Sclerotinia sclerotiorum* is a notorious pathogen with a broad host range, capable of infecting over 400 plant species (Bolton et al., 2006). In contrast, *C. shiraiana* had a narrow host range, infecting plants in the *Moraceae* family (Zhu et al., 2021). In agricultural production, there are few effective methods to control this disease. Therefore, identifying key elicitors and understanding molecular mechanisms may be promising for developing novel strategies to control Sclerotinia diseases.

In this study, it was demonstrated that the novel elicitor Cs08297 from *C. shiraiana* could induce cell death in various plants. The ability of Cs08297 to induce cell death was mediated by leucine-rich repeat (LRR) receptor-like kinases BAK1 and SOBIR1 in *N. benthamiana*. Cs08297 triggered plant defence responses such as ROS burst, callose accumulation and expression of defence-related genes. Treatment of plants with His-tagged Cs08297-thioredoxin fusion protein also enhanced resistance against *S. sclerotiorum* and *C. shiraiana*.

2 | RESULTS

2.1 | Cs08297 is an elicitor inducing plant cell death

The effectors of *C. shiraiana* were previously screened (Zhang et al., 2024). To identify elicitors that could induce cell death, the transient expression of these proteins was carried out in *N. benthamiana*. Among them, Cs08297 induced cell death in *N. benthamiana* (Figure 1a). Cell death was not induced in GFP control group (Figure 1a). Next, the electrolyte leakage and HR-specific marker gene expression levels were examined in the agroinfiltrated leaves. The electrolyte leakage and gene expression levels were higher in leaves agroinfiltrated with the Cs08297 construct than those in leaves agroinfiltrated with the GFP construct (Figure 1b,c), indicating that Cs08297 can induce plant cell death. To further confirm this, Cs08297 was expressed in *Escherichia coli* and then purified (Figure 2a). To investigate whether cell death induction by Cs08297 is species specific, His-tagged Cs08297-thioredoxin fusion protein was injected into the leaves of mulberry, tobacco, tomato, and strawberry. His-tagged Cs08297-thioredoxin fusion protein caused plant cell death in various plants (Figure 2b). The purified His-tagged Cs08297-thioredoxin fusion protein was infiltrated into *N. benthamiana* and mulberry leaves in concentrations from 10 ng/mL to 100 μg/mL. As shown in Figure 2c, the concentration of His-tagged Cs08297-thioredoxin fusion inducing cell death in *N. benthamiana* was 100 μg/mL, while in mulberry this was 50 μg/mL. Thus, Cs08297 is an elicitor inducing plant cell death.

2.2 | The apoplastic space is necessary for Cs08297 inducing cell death

According to bioinformatics analysis, Cs08297 could act as a secreted protein with a signal peptide (SP) located at its N-terminus (1–23 amino acids, aa) (Figure 1a). To further analyse whether this SP was associated with Cs08297 inducing cell death, Cs08297 lacking the SP (Cs08297$^{\Delta SP}$) was transiently expressed in *N. benthamiana*. Cs08297$^{\Delta SP}$ did not induce cell death in *N. benthamiana* (Figure 1a). Electrolyte leakage and HR-specific marker gene expression levels were consistent with the above phenotype (Figure 1b,c). Therefore, the SP is important for Cs08297 function. pCAMBIA1300-Cs08297-GFP and pCAMBIA1300-Cs08297$^{\Delta SP}$-GFP constructs were agroinfiltrated in *N. benthamiana* to investigate the subcellular localization of Cs08297. Cs08297, but not Cs08297$^{\Delta SP}$, was observed to be localized on plant membranes as well as in the extramembrane region after plasmolysis (Figure S1a,b). To further confirm that Cs08297 is localized in the plant apoplast, apoplastic fluid (AF) in *N. benthamiana* was collected from agroinfiltrated plants. Western blotting analysis

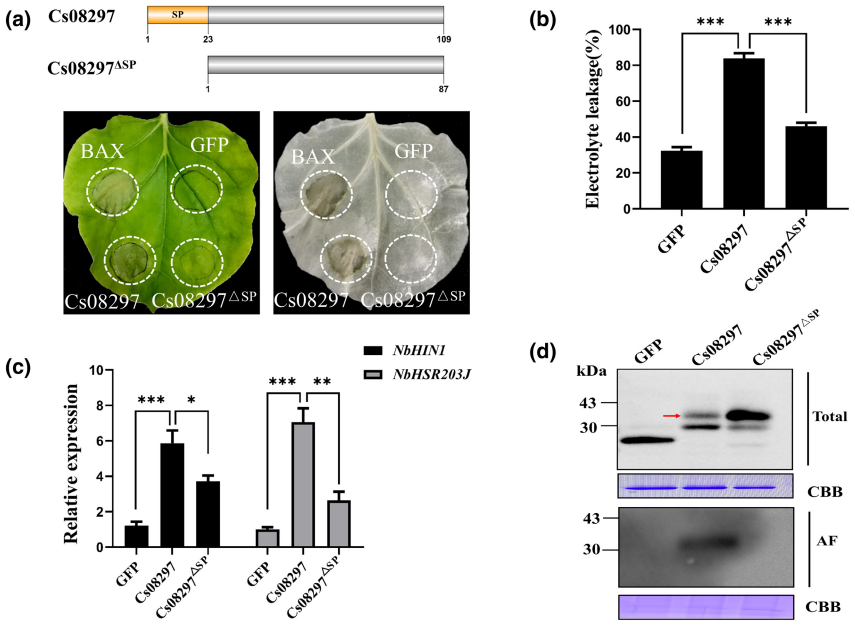

FIGURE 1 Cs08297 is an elicitor inducing cell death in *Nicotiana benthamiana* leaves. (a) Transient expression of Cs08297 and Cs08297$^{\Delta SP}$ in *N. benthamiana* mediated by *Agrobacterium tumefaciens*. Photographs were taken 7 days post-agroinfiltration (dpa). In the schematic diagram of Cs08297 protein structure (above), the orange box represents the signal peptide (SP). Observation of necrotic spots (below left) by and after decolourization (below right). (b) Cell necrosis index was measured in leaves agroinfiltrated with different constructs at 7 dpa. Six leaf discs (9 mm diameter) were measured in each group. The experiment was repeated three times. (c) The expression levels of hypersensitive response-related genes were analysed in *N. benthamiana* leaves. Three leaves were counted in each replicate. The experiment was repeated three times. (d) Western blot analysis of total or apoplastic extractions from *N. benthamiana* leaves transiently expressing *Ciboria shiraiana* Cs08297. Expected protein band is indicated by the red arrow. AF indicates apoplastic fluid. Coomassie brilliant blue (CBB) staining was used to indicate equal loading in each sample. Error bars represent ± *SEM* (Student's *t* test, $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$).

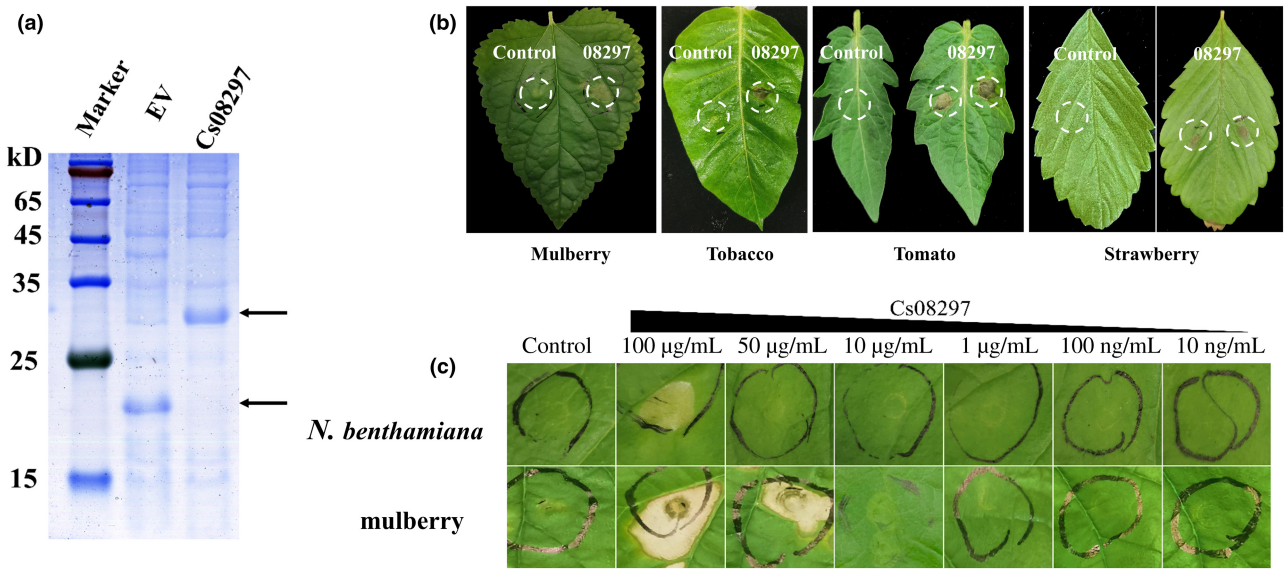

FIGURE 2 Prokaryotic expression of His-tagged Cs08297-thioredoxin fusion protein could induce cell death in various plants. (a) Identification of purified recombinant protein His-tagged Cs08297-thioredoxin fusion protein by SDS-PAGE. The two black arrows indicate the bands corresponding to the target proteins. (b) Treatment of mulberry (*Morus alba* 'Guiyou No. 62'), tobacco (*Nicotiana tabacum*), tomato (*Solanum lycopersicum*), and strawberry (*Fragaria vesca*) leaves with 100 μg/mL purified His-tagged Cs08297-thioredoxin fusion protein and His-tagged Cs08297-thioredoxin as a control. Treated leaves of different plants were photographed 72 h post-infiltration (hpi). The white circle in the leaves represents the infiltration area. (c) Different concentrations of His-tagged Cs08297-thioredoxin fusion protein were infiltrated into *N. benthamiana* and mulberry leaves, then cell death was observed and photographed 72 hpi.

showed that Cs08297 was detectable in both AF and total protein extracts, while Cs08297$^{\Delta SP}$ was only detectable in total protein extracts (Figure 1d). Taken together, the apoplastic space is crucial for Cs08297 inducing cell death.

2.3 | Cs08297 activates plant defence responses and enhances plant resistance

To determine whether the cell death triggered by Cs08297 is associated with plant immune responses, ROS accumulation, callose deposition, and the expression levels of genes related to the PTI pathway in *N. benthamiana* were examined. In Cs08297-treated leaves, the accumulation of H_2O_2 and callose deposition were significantly higher than those in the GFP control leaves (Figure 3a,b). The expression levels of genes related to the PTI pathway were upregulated in the leaves agroinfiltrated with the Cs08297 construct (Figure 3c). His-tagged Cs08297-thioredoxin fusion protein was also infiltrated in mulberry leaves and expression levels of defence-related genes were examined. As shown in the Figure S2, compared to the His-tagged thioredoxin protein, the expression levels of defence-related genes in mulberry were upregulated in the Cs08297-treated leaves. These results suggested that Cs08297 can activate plant immune responses.

To clarify whether the immunity activated by Cs08297 was accompanied by alteration of hormone signalling pathways, the expression levels of hormone signalling-related defence genes were investigated. The expression patterns of marker genes associated with (i) salicylic acid (SA)-dependent immune pathways, such as *NbPR1a* and *NbPR2*, (ii) jasmonic acid (JA)-dependent immune pathways, including *NbPR4* and *NbLOX*, and (iii) ethylene-dependent immunity, *NbERF1* were examined. As shown in Figure 3c, compared to the control group, the expression levels of *NbPR1a*, *NbPR2*, *NbPR4*, and *NbLOX* were significantly upregulated in Cs08297-injected leaves, while the expression of *NbERF1* was not significantly changed. These findings indicate that Cs08297 could trigger immunity by activating the SA- and JA-mediated defence pathways. To study whether Cs08297 could improve plant disease resistance against phytopathogenic fungi, *C. shiraiana* and *S. sclerotiorum* were inoculated on *N. benthamiana* leaves treated with His-tagged Cs08297-thioredoxin fusion, and the lesion areas were measured (Figure 3d,e). The results showed that compared to the control, *C. shiraiana* and *S. sclerotiorum* caused smaller lesions and reduced pathogen biomass on His-tagged Cs08297-thioredoxin fusion-infiltrated leaves (Figure 3d,e). Thus, Cs08297 can enhance resistance against pathogens in plants.

2.4 | BAK1 and SOBIR1 are required for Cs08297-induced cell death in *N. benthamiana*

PRRs are commonly employed by plants to identify PAMPs secreted by pathogenic bacteria and to activate signalling pathways. Notably, BAK1 and SOBIR1 serve as co-receptors for numerous PRRs, triggering signal transduction cascades upon the recognition of various PAMPs. To determine whether Cs08297-induced cell death was dependent on BAK1 and/or SOBIR1, virus-induced gene silencing

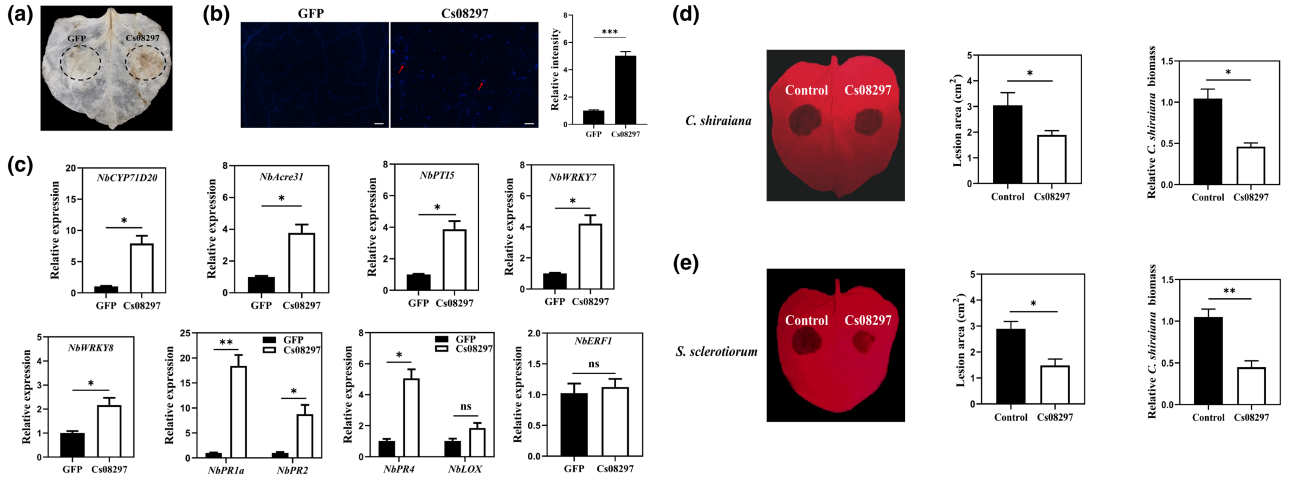

FIGURE 3 Cs08297 can trigger plant immune responses and enhance plant resistance in *Nicotiana benthamiana*. (a) Accumulation of reactive oxygen species (ROS) and (b) deposition of callose in *N. benthamiana* at 48 h post-agroinfiltration (dpa). The bars represent 200 μm. The relative fluorescence intensity was analysed using ImageJ software. (c) The relative expression levels of PAMP-triggered immunity (PTI) marker genes and hormone-related defence genes were determined in *N. benthamiana* leaves infiltrated with Cs08297 construct at 2 dpa. Three leaves were counted in each replicate. The experiment was repeated three times. Phenotypes of treated *N. benthamiana* leaves inoculated with *Ciboria shiraiana* (d) and *Sclerotinia sclerotiorum* (e) at 24 h post-inoculation under UV light. *N. benthamiana* leaves were treated with 50 μg/mL purified His-tagged Cs08297-thioredoxin fusion protein and then inoculated with mycelium plugs of *C. shiraiana* and *S. sclerotiorum*. His-tagged-thioredoxin was used as a control. Lesion areas were assessed from three independent experiments ($n = 9$). The fungal biomass in *N. benthamiana* were determined using PCR. Error bars represent ± *SEM* (Student's *t* test, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$; ns: not significant).

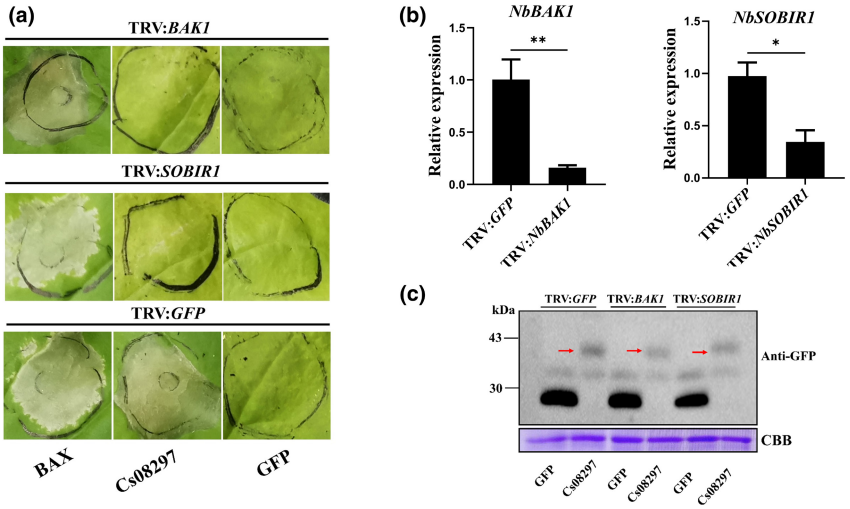

FIGURE 4 Cs08297-triggered cell death in *Nicotiana benthamiana* requires BAK1 and SOBIR1. (a) BAK1 and SOBIR1 were indispensable for Cs08297-induced cell death in *N. benthamiana*. Cs08297, BAX, and GFP were transiently expressed in the silenced plants. Photographs were taken 7 days post-agroinfiltration (dpa). (b) The expression levels of target genes analysis in different silenced lines. Three leaves were counted in each replicate. The experiment was repeated three times. (c) Immunoblot analysis for green fluorescent protein (GFP) and Cs08297 fused with GFP tag expressed in silenced strains. Expected protein bands were indicated by red arrows. Coomassie brilliant blue (CBB) staining was used to indicate equal loading in each sample. Error bars represent ± *SEM* (Student's *t* test, $^{*}p < 0.05$, $^{**}p < 0.01$).

(VIGS) constructs were generated to silence *NbBAK1* and *NbSOBIR1* in *N. benthamiana*. BAX, GFP, and Cs08297 constructs were transiently expressed in these silenced plants. The results indicated that Cs08297 did not trigger cell death in BAK1- and SOBIR1-silenced plants, but it could induce cell death in TRV:GFP-silenced plants (Figure 4a). As a positive control, BAX induced cell death in all silenced plants. Immunoblotting analysis confirmed that Cs08297 was successfully expressed in all silenced plants (Figure 4c). Moreover, reverse transcription quantitative-PCR (RT-qPCR) analysis revealed that the relative expression levels of *NbBAK1* and *NbSOBIR1* were reduced to approximately 30% in their respective silenced plants compared to the TRV:GFP-silenced plant (Figure 4b). These results suggest that BAK1 and SOBIR1 are required for Cs08297-induced cell death in *N. benthamiana*.

2.5 | Cs08297 is a cysteine-rich effector unique to *C. shiraiana*, and the cell death-inducing ability of Cs08297 is dependent on its cysteine residues

Cs08297 contains 109 amino acid residues, including six cysteine residues (Figure 5a). Bioinformatics analysis indicated that no homologous protein of Cs08297 was found in plants, bacteria, fungi, and oomycetes, suggesting that Cs08297 is a cysteine-rich effector unique to *C. shiraiana*. Disulphide bonds can be formed between two cysteine residues to maintain the stability of the protein structure, and the spatial structure of the protein affects its function. To determine whether the cysteine residues affect the ability of Cs08297 to induce plant cell death, site-directed mutations of all six cysteine residues was created and the mutant proteins were then expressed in *N. benthamiana* by *Agrobacterium tumefaciens*-mediated transient expression. The results showed that none of the six proteins with a mutated cysteine residue caused plant cell death (Figure 5b). Immunoblotting analysis confirmed that all six mutant proteins were normally expressed in *N. benthamiana* (Figure 5c). These results indicates that these six cysteine residues are essential for Cs08297 to induce plant cell death, implying that the spatial structure of Cs08297 is important to the induction of plant cell death.

2.6 | Cs08297 is required for *C. shiraiana* sclerotial development, oxidative-stress adaptation, and cell wall integrity

To explore the function of *Cs08297* in *C. shiraiana*, *Cs08297* was knocked down using the RNA interference (RNAi) method. Two independent *Cs08297*-silenced strains were obtained; the relative expression levels of *Cs08297* were down-regulated to 20%–50% in these silenced strains (Figure S3). The colony diameters of wild-type (WT) and RNAi strains were measured on the potato dextrose agar (PDA) plates. There was no significant difference in hyphal growth between the WT and *Cs08297*-silenced strains after 24 and 48 h of cultivation (Figure S4a,b). After 14 days of cultivation, the number of sclerotia in the RNAi strains was decreased to about 65% compared to the WT strain, while the average sclerotial weight increased by 59% compared to the WT strain (Figure S4c–e). To investigate whether *Cs08297* is involved in the stress responses, the hyphal growth rates of WT and silenced strains were measured on PDA containing various stress-inducing agents. There was no significant difference in the growth and inhibition rate of hyphal growth between WT and silenced strains on PDA containing NaCl and KCl (osmotic stress) (Figure S5a). However, in PDA containing hydrogen peroxide (oxidative stress) and sodium dodecyl sulphate (cell wall integrity stress), the growth of hyphae in silenced strains was suppressed, and the inhibition of hyphal growth reached about 50% and 65%, respectively (Figure S5b). Therefore, Cs08297 is required for sclerotial development and oxidative-stress response and cell wall integrity in *C. shiraiana*.

2.7 | *Cs08297* negatively regulates *C. shiraiana* virulence

To evaluate the role of *Cs08297* on the virulence of *C. shiraiana*, the expression profile of *Cs08297* during infection was determined using reverse transcription-quantitative PCR (RT-qPCR). The expression level of *Cs08297* in the WT increased by 100-fold at 12 h post-infection (hpi), implying *Cs08297* is involved in pathogenesis (Figure S6). Subsequently, the lesion areas on the leaves caused by the WT and silenced strains were measured. Compared with the

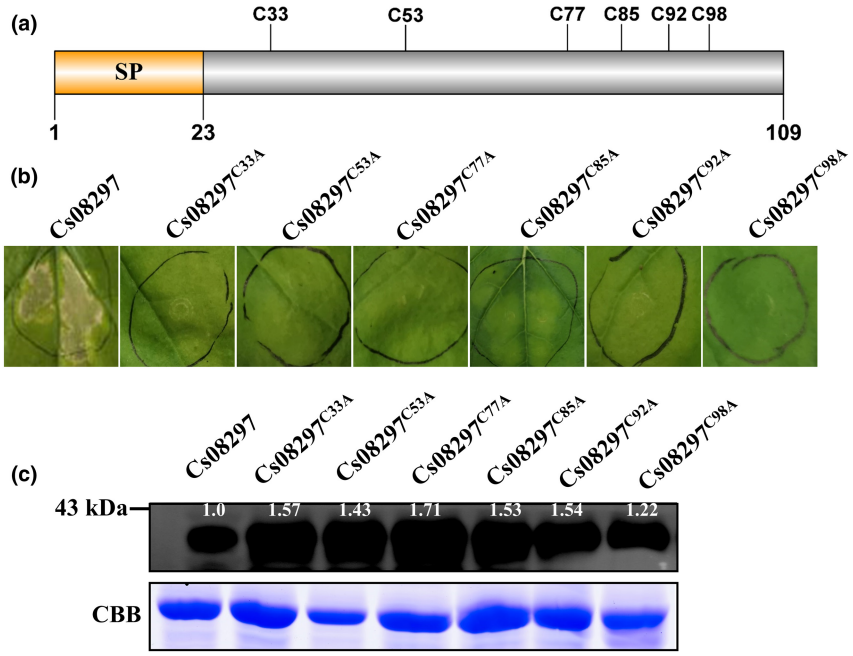

FIGURE 5 The cysteine residues in Cs08297 affect its ability to induce cell death. (a) Schematic diagram showed the distribution of cysteine residues in Cs08297 protein. (b) Cell death-inducing activity of the mutant proteins was assessed 7 days post-agroinfiltration (dpa) by transient expression in 4-week-old *Nicotiana benthamiana* leaves. (c) Immunoblot analysis of all mutant proteins transiently expressed in *N. benthamiana* leaves. The protein was quantitatively analysed by ImageJ software. Coomassie brilliant blue (CBB) staining is used to indicate equal loading in each sample.

WT strain, *Cs08297*-silenced strains caused larger lesion areas on *N. benthamiana* and mulberry leaves, and the biomass of *C. shiraiana* in *N. benthamiana* was consistent with this (Figure 6). These results suggested that *Cs08297* is a negative regulator of *C. shiraiana* virulence.

3 | DISCUSSION

It is widely acknowledged that effectors play a pivotal role in the pathogenicity of pathogens to host plants (Lovelace et al., 2023). Effectors are helpful for pathogens to establish a favourable environment, including countering plant immunity, modifying the host metabolism and physiology, and inducing cell death (Friesen & Faris, 2021). The multifaceted function of elicitors underscores their importance in interactions between pathogens and host plants, and as determinants for pathogenicity. In this present study, an apoplastic effector Cs08297 was discovered and found to trigger plant immunity and induce plant cell death. Moreover, His-tagged Cs08297-thioredoxin fusion protein could enhance plant resistance to pathogens.

The apoplastic space serves as a complex battlefield where a large number of crucial interactions between plants and pathogens occurs (Mott et al., 2014). In this research, Cs08297 was found to induce cell death in the presence of a SP, which was abolished in the absence of the SP (Figure 1a). This is similar to the case of FoEG1, a cell death elicitor from *Fusarium oxysporum* (Zhang et al., 2021a). The results showed that Cs08297 localized to the plant apoplast (Figures 1 and S1). The apoplastic space is often disrupted by pathogens to deconstruct plant integrity and then facilitate pathogenicity. In response, plants produce defence molecules that could slow the spread of pathogens and activate the plant immune response (Dodds & Rathjen, 2010). For example, plants rapidly produce ROS as signalling molecules to regulate downstream pathways (Qi et al., 2017). ROS can also promote plant cell death, which resists the invasion of pathogens to a certain extent. Around the site of pathogen infection, callose deposits are promptly accumulated in plants to restrict the penetration and growth of pathogens (Luna et al., 2011). The apoplastic space was found to be crucial for the function of Cs08297. The expression of Cs08297 in *N. benthamiana* activated plant basal defences, encompassing ROS production, callose deposition, and upregulation of a set of resistance genes involved in SA- and JA-signalling pathways (Figure 3). His-tagged Cs08297-thioredoxin fusion treatment also upregulated PTI-related marker genes in mulberry leaves

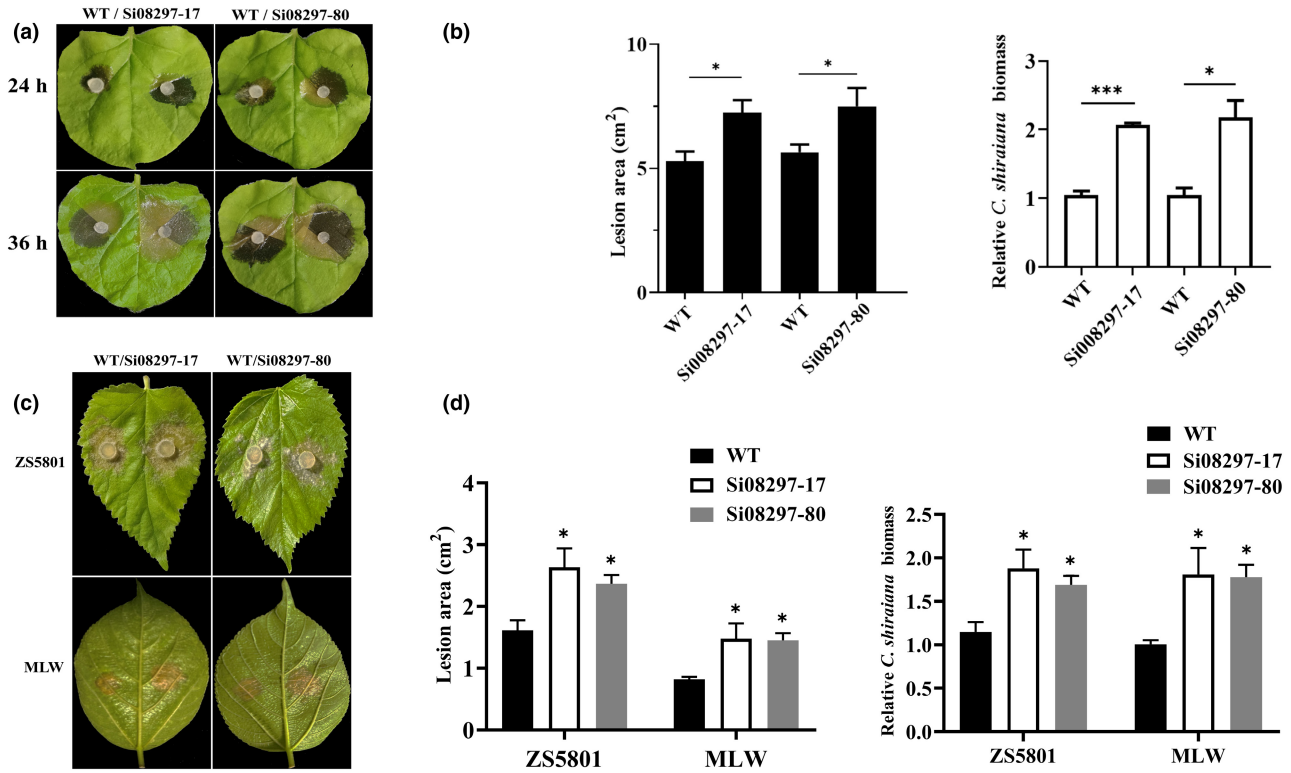

FIGURE 6 Knockdown of *Cs08297* promoted the virulence of *Ciboria shiraiana*. (a) Representative *Nicotiana benthamiana* leaves showing lesions caused by *Cs08297*-silenced and wild-type (WT) strains. Photographs were taken at 24 and 36 h post-inoculation (hpi). Lesion areas were assessed from three independent experiments ($n = 9$). (b) Lesion diameters and fungal biomass were measured in *N. benthamiana* leaves. The fungal biomass of WT and silenced strains in *N. benthamiana* were determined using PCR. (c) Lesion areas of mulberry leaves caused by *Cs08297*-silenced and WT strains. MLW stands for resistant mulberry *Morus laevigata* and ZS5801 is susceptible mulberry *Morus alba* 'ZS5801'. Lesion areas were assessed from three independent experiments ($n = 9$). (d) Lesion diameters and fungal biomass were measured in mulberry leaves. The fungal biomass of WT and silenced strains in *N. benthamiana* were determined using PCR. Error bars represent ± *SEM* (Student's *t* test, $^{*}p < 0.05$. $^{***}p < 0.001$).

(Figure S2). We suggest that the function of Cs08297 is similar to that of PAMPs and is important in inducing plant immunity.

BAK1 and SOBIR1 are receptors located on the plasma membrane, which form complexes with various PRRs to enhance plant perception of pathogens, thereby activating immune responses (Liang & Zhou, 2018). In *N. benthamiana*, RXEG1 specifically recognizes XEG1 from *P. sojae* through its an amino-terminal and a carboxy-terminal loop-out region, and recruits co-receptors BAK1 and SOBIR1 to form a PRR complex to transduce XEG1-induced defence signals (Sun et al., 2022; Wang et al., 2018). Similar to XEG1, cell death triggered by VdEG1, VmE02, and Fg02685 is dependent on BAK1 and SOBIR1 (Gui et al., 2017; Nie et al., 2021; Xu et al., 2022). In contrast, SGP1 from *Ustilaginoidea virens* triggers cell death that is only dependent on BAK1, but not on SOBIR1 (Song et al., 2021). This study showed that Cs08297 did not trigger cell death in *NbBAK1*-silenced and *NbSOBIR1*-silenced plants, indicating Cs08297 induction of plant cell death is dependent on BAK1 and SOBIR1 (Figure 4a). BAK1 and SOBIR1 have been demonstrated to typically form ternary complexes with RLP, collectively transmitting immune signals to downstream components (Li et al., 2020). Thus, we speculate that Cs08297 might be recognized in plants by the LRR-RLPs/SOBIR1/BAK1 complex. However, the specific receptor to Cs08297 and corresponding recognition mechanism in plants were unclear and require further investigation. Many cell death-inducing proteins contain key sites that play a critical role in cell death induction or plant immune response activation. For example, four conserved sites (D112, H120, D123, and E125) in PcNLP1 from *Phytophthora capsici* are essential for inducing cell death in pepper and tobacco leaves (Feng et al., 2014). Two cysteine residues in BcXYG1, a xyloglucanase secreted from *Botrytis cinerea*, are important for inducing cell death (Zhu et al., 2017). In this study, we found that Cs08297 is a cysteine-rich effector, and that six cysteine residues in Cs08297 are crucial for its ability to induce cell death (Figure 5b). Disulphide bonds can be formed between cysteines in proteins, which are essential for protein stability and maintenance of structure. Therefore, we hypothesized that the ability of Cs08297 to induce cell death may be related to the spatial structure of the protein, which needs to be further explored.

Sclerotia can survive for several years in harsh biological and physical environments, including low temperatures and dry conditions. Therefore, in agricultural fields, they are crucial for the survival and persistence of infectious propagules. When under favourable environmental conditions, sclerotia germinate to form an apothecium and a large number of ascospores are released to initiate a new infectious cycle (Zhang, Zhu, et al., 2021). Here, in this study, it was found that *Cs08297*-silenced strains produced fewer sclerotia, but there was a significant increase in the weight of individual sclerotial weight compared to that of the WT strain (Figure S4d,e). We speculated that Cs08297 might be involved in the regulation of cell division and proliferation. The downregulated expression of *Cs08297* led to a decrease in cell division and also the number of sclerotia. On the other hand, nutrient uptake and utilization by fungi might be influencing factors. Interestingly, our results demonstrated that *Cs08297* acted as a negative regulator of *C. shiraiana* virulence (Figure 6). A similar function has been observed in effectors from other fungi. For instance, knocking out the virulence gene *Ave1* enhanced the virulence of *Verticillium dahliae* on *Ve1* tomato (de Jonge et al., 2012). Likewise, deletion of the apoplastic effector PcEXLX1 from *P. capsici* resulted in increased virulence of the mutants in *N. benthamiana* (Pi et al., 2022). We speculate that the increased susceptibility of plants to *Cs08297*-silenced mutants may result from the loss of Cs08297 recognition and the subsequent failure to activate the plant immune response. Additionally, researchers have found that microRNA-like RNAs can silence effector-coding gene expression to evade host defence activation, thereby finely regulating the pathogenicity of *Fusarium oxysporum* f. sp. *cubense* (Li et al., 2022). A similar mechanism may be at play in Cs08297 of *C. shiraiana*, which needs to be further explored.

Mulberry sclerotinia disease causes severe yield losses worldwide. There have been reports about genes related to disease resistance genes, such as *WRKY53*, *RGA3*, and *RPM1-2* (Fan et al., 2023; Negi & Khurana, 2021). However, due to the complexity of plant resistance mechanisms and limitations in transgenic technology, there are difficulties in development of disease-resistant mulberry varieties. Additionally, the excessive use of traditional fungicides has contributed to environmental pollution and increased resistance in pathogens. Therefore, research on environment-friendly biological control methods provides a broader perspective. Disease resistance in rice and *N. benthamiana* has been induced by foliar application of SNP22 (Song et al., 2021). A 32-amino acid peptide, FgNP32, derived from the effector protein Fg02685 in *Fusarium graminearum* was recently identified as a plant immune inducer that enhances plant resistance to Fusarium head blight through the PTI pathway (Xu et al., 2022). In our study, Cs08297 from *C. shiraiana* was identified as an elicitor and induced plant immunity. Treatment with exogenous His-tagged Cs08297-thioredoxin fusion protein in *N. benthamiana* contributed to plant resistance against *S. sclerotiorum* and *C. shiraiana* (Figure 3d,e). From this point of view, this could provide a reference for controlling crop diseases.

In summary, a novel protein elicitor Cs08297 from *C. shiraiana* was identified that could induce plant immune responses. Cs08297 is an apoplastic effector and triggers cell death dependent on BAK1 and SOBIR1 in *N. benthamiana*. Moreover, treatment with His-tagged Cs08297-thioredoxin fusion enhanced *N. benthamiana* resistance against pathogens. The results could help to elucidate the molecular mechanisms of Cs08297 triggering plant immunity. Cs08297 is anticipated to emerge as a novel biological fungicide to mitigate the impact of diseases and enhance the production efficiency of agriculture.

4 | EXPERIMENTAL PROCEDURES

4.1 | Culture conditions, DNA isolation

N. benthamiana and mulberry (*Morus alba* 'Guiyou No. 62') were grown in chambers under controlled conditions (day/night 25°C/20°C; 16 h light/8 h dark; RH 60%). *C. shiraiana* WCCQ01 and *S. sclerotiorum* 1980

were cultivated on PDA at 25°C. Mulberry leaves from resistant (*Morus laevigata*) and susceptible varieties (*M. alba* 'Zhongsang 5801') used in infection experiments were collected from the mulberry orchard at Southwest University, China. Genomic DNA from plant and fungi were extracted using the genomic DNA kit (TIANGEN Biotech) following the manufacturer's instructions.

4.2 | RNA isolation and gene expression analysis

The collected samples are immediately placed in liquid nitrogen and stored at −80°C for further use. Total RNA was extracted using the EZ-10 Total RNA Mini-Preps Kit (Sangon Biotech) according to the manufacturer's protocol. The RNA was reverse transcribed into cDNA using PrimeScript RT Reagent Kit (TaKaRa). Quantitative PCR (qPCR) was performed using PrimeScript RT reagent Kit (Perfect Real Time) (TaKaRa). The transcript abundances of genes were analysed through RT-qPCR. The *β-actin* gene was used as internal control and the relative expression levels of genes were calculated using the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001).

4.3 | Plasmid construction

For transient gene expression in *N. benthamiana*, Cs08297 and Cs08297$^{\Delta SP}$ (without SP) were ligated with potato virus X (PVX) vector pGR106 (Wagner et al., 2004) digested with specific enzymes (ClaI and SalI for PVX vector). To generate a construct for prokaryotic expression, the coding region of Cs08297 (SP removed) was ligated into the pET-32a vector digested with BamHI and SacI. To analyse the subcellular localization of Cs08297 in plants, Cs08297 and Cs028297$^{\Delta SP}$ were cloned into the pCambia1300-GFP vector digested with SacI and KpnI. For RNAi experiments in *C. shiraiana*, the sense and antisense silencing fragments of *Cs08297* were ligated into a pSilent-1 vector (Nakayashiki et al., 2005) digested with XhoI, HindIII, KpnI, and BglII. For VIGS in *N. benthamiana*, the silencing fragments of *NbBAK1* and *NbSOBIR1* were cloned into TRV2 vector digested with BamHI and EcoRI. All constructs were sequenced to verify their accuracy (Sangon Biotech). The primers used in this study are listed in Table S1.

4.4 | Protein expression and western blot analysis

To create constructs for recombinant protein, the coding region of *Cs08297* (excluding the SP) was inserted into the pET-32a vector. The recombinant protein was expressed in *Escherichia coli* BL21(DE3), induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at 16°C for 24 h. After induction, cells were collected through centrifugation and resuspended in phosphate-buffered saline (PBS, pH 7.5). The suspended cells were lysed via sonication, and the supernatant was obtained by centrifugation at 10,000*g* for 10 min. The supernatant was purified using affinity chromatography using Ni-NTA resin (Sangon Biotech) following the manufacturer's instructions. The target protein was eluted with 50 mM imidazole, and the elution samples were dialysed overnight at 4°C without agitation using a Spectrum/Por dialysis membrane with molecular weight cut-off (MWCO) of 3500 (Beyotime Biotech), during which the buffer was changed several times. Protein concentration was determined using the BCA protein concentration assay kit (Beyotime Biotech).

Total protein was extracted using RIPA lysis buffer consisting of 1 mM phenylmethanesulfonyl fluoride and protease inhibitor cocktail. The proteins were separated by 12% SDS-PAGE, transferred to a polyvinylidene fluoride membrane, and detected with a specific anti-GFP antibody (Proteintech Group). Horseradish peroxidase-conjugated goat anti-mouse IgG (H + L) antibody was used to identify the primary specific antibody. Protein bands were detected using ECL substrate following the manufacturer's instructions.

4.5 | Transient expression in *N. benthamiana*

For transient expression in *N. benthamiana*, Cs08297 and Cs08297$^{\Delta SP}$ were separately cloned into the PVX vector pGR106. *A. tumefaciens* GV3101 strains containing the individual recombinant plasmids were cultured overnight at 28°C in LB medium with appropriate antibiotics. The cells were washed three times with infiltration buffer (10 mM $MgCl_2$, 10 mM MES, 200 μM acetosyringone, pH 5.7) and resuspended to OD_{600} = 0.4–0.8. The cell suspension was injected into leaves of 4- to 6-week-old *N. benthamiana* plants with a needleless syringe. To analyse the subcellular localization of Cs08297 in planta, Cs08297 and Cs08297$^{\Delta SP}$ were cloned into pCambia1300 vector. The recombinant plasmids were transformed into *A. tumefaciens* GV3101 and agroinfiltrated into 4-week-old *N. benthamiana* leaves. At 2–3 days post-agroinfiltration, GFP localization was observed using a FV1200 confocal laser scanning microscope (Olympus). Plasmolysis was induced treating leaves with 30% sucrose for 10 min.

Constructs for VIGS in *N. benthamiana* were generated in the TRV2 vector, using a *N. benthamiana* cDNA library for gene fragment amplification. For VIGS assays in *N. benthamiana*, the plasmid vectors pTRV1 and pTRV2 with target genes were transformed into *A. tumefaciens* GV3101. The cell suspensions containing TRV2 vectors were combined with *A. tumefaciens* cell suspension carrying TRV1 in a 1:1 ratio before infiltration. The cell suspensions were then infiltrated into three primary leaves of four-leaf-stage *N. benthamiana* plants. After 3 weeks of agroinfiltration, the silencing efficiency of the target genes were analysed by RT-qPCR.

4.6 | Apoplast fluid isolation

The apoplast fluid (AF) of *N. benthamiana* leaves was collected by the infiltration-centrifugation method as described previously (Xu et al., 2021). The agroinfiltrated leaves were soaked with PBS and

placed in a vacuum pump. The vacuum was released slowly and the PBS was pushed into the leaf apoplast. The leaf surface was dried to remove the remaining PBS and then put into a 10 mL syringe without a needle for centrifugation at 1000 *g* for 10 min at 4°C. Finally, the AF was collected into a 50 mL centrifuge tube.

4.7 | Defence responses detection in *N. benthamiana*

To measure ROS in plants, leaves were cut and immersed in a solution containing 1 mg/mL 3,3′-diaminobenzidine (DAB) and soaked for 12 h, then decolourized with ethanol. The final brown colour of the leaves is an indicator of ROS accumulation. Callose deposition in the leaves was detected by aniline blue staining as described previously (Shangguan et al., 2018). Stained samples were viewed under an IX71 fluorescent microscope (Olympus). To explore the expression of defence-related genes in leaves, we collected total RNA from the treated leaves and RT-qPCR was performed. The primers are listed in Table S1.

4.8 | Electrolyte leakage assay

Four-week-old *N. benthamiana* leaves were infiltrated with cultures of *A. tumefaciens* GV3101 carrying different constructs. The leaves were collected 7 days after agroinfiltration and rinsed at room temperature using distilled water. The conductivity of solution was measured using a conductivity meter (FiveEasyPlus; Mettler-Toledo) to produce value A. Then, the leaf disks were boiled in the bathing solution and the conductivity was measured to yield value B. The percentage electrolyte leakage was calculated as (A/B) × 100.

4.9 | *C. shiraiana* transformation and pathogen inoculation assays

To generate the *Cs08297* knockdown strains, an RNAi construct was transferred into the protoplasts of *C. shiraiana* by a polyethylene glycol-mediated method. The knockdown mutants were screened on plates containing hygromycin and identified by PCR using specific primers. The expression levels of target genes in the transformants were measured using RT-qPCR. For pathogen inoculation assays, a 9 mm mycelial plug from the colony edge was inoculated on *N. benthamiana and* mulberry leaves. The diameters of lesions were measured at various time points and each experiment was repeated three times.

4.10 | Statistical analysis

The data were reported as mean ± *SEM* from three independent biological replicates and analysed with GraphPad Prism 8 software. Significant differences between mean values were evaluated using Student's *t* test ($p < 0.05$). Asterisks are used to indicate $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***).

ACKNOWLEDGEMENTS

This work was supported by the Hainan Province Science and Technology Special Fund (ZDYF2022SHFZ319), the earmarked fund for China Agriculture Research System (CARS-18-ZJ0201), the Chongqing Modern Agricultural Industry Technology System (CQMAITS202311), and the Chongqing Talent Program (cstc2024ycjh-bgzxm0086).

CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request. Sequence data for Cs08297 is available at GenBank at https://www.ncbi.nlm.nih.gov/genbank/ with accession number WLS32029.1.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Zhang, S., Li, R., Fan, W., Chen, X., Tao, C., Liu, S. et al. (2024) A novel protein elicitor (Cs08297) from *Ciboria shiraiana* enhances plant disease resistance. *Molecular Plant Pathology*, 25, e70023. Available from: https://doi.org/10.1111/mpp.70023