A prophage-encoded nonclassical secretory protein of “Candidatus Liberibacter asiaticus” induces a strong immune response in Nicotiana benthamiana and citrus

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Abstract
Huanglongbing (HLB), associated with “Candidatus Liberibacter asiaticus” (CLas), is a globally devastating plant disease. The highly reduced genome of CLas encodes a number of secretory proteins. The conserved prophage-encoded protein AGH17470 is herein identified as a nonclassical secretory protein. We confirmed that the N-terminal and C-terminal sequences jointly determine the secretion of AGH17470. The transient expression of AGH17470 protein in Nicotiana benthamiana caused hypersensitive response (HR) cell death in infiltrated leaves and systemically infected leaves as well as the dwarfing of the entire plant, suggesting that AGH17470 is involved in the plant immune response, growth, and development. Overexpression of AGH17470 in N. benthamiana and citrus plants up-regulated the transcription of pathogenesis-related and salicylic acid (SA)-signalling pathway genes and promoted SA accumulation. Furthermore, transient expression of AGH17470 enhanced the resistance of sweet orange to Xanthomonas citri subsp. citri. To our knowledge, AGH17470 is the first prophage-encoded secretory protein demonstrated to elicit an HR and induce a strong plant immune response. The findings have increased our understanding of prophage-encoded secretory protein genes, and the results provide clues as to the plant defence response against CLas.

KEYWORDS
CLas, huanglongbing (HLB), prophage, secretory protein, virulence factor

1 | INTRODUCTION

Huanglongbing (HLB) is a devastating plant disease that hampers the development of the worldwide citrus industry (Bove, 2006; Wang, 2019). The probable pathogen associated with HLB is a gram-negative bacterium belonging to the α-proteobacterium “Candidatus Liberibacter” group that can be divided into three species: “Ca. L. asiaticus” (CLas), “Ca. L. africanus”, and “Ca. L. americanus” (Jagoueix et al., 1994; Teixeira et al., 2005). Among the three species, CLas is the most prevalent and aggressive in the major global citrus-producing regions (Wang et al., 2017). Although research on CLas has attracted significant attention, the pathogenicity mechanism of CLas remains largely unknown due to the lack of pure cultures of CLas.

As an important component of pathogens, secretory proteins (also termed effectors) of fungi, bacteria, and oomycetes play critical roles in the evolutionary arms race between plants and pathogens, and they contribute to disease pathogenesis (Kale et al., 2010;
Lindeberg et al., 2012; Tyler et al., 2006). CLas lacks the type III (T3SS) and type IV secretion systems (T4SS) that are common in gram-negative bacteria (Abramovitch et al., 2006). However, CLas harbours a type I secretion system (T1SS) and Sec-dependent secretory machinery that confer the capability to release secretory proteins (Duan et al., 2009). Transient expression of Las5315 (SDE1) induces cell death, strong callose deposition, and massive starch accumulation in Nicotiana benthamiana (Marco et al., 2016, 2018). Furthermore, SDE1 significantly promotes CLas infection and enhances HLB symptoms in citrus plants (Clark et al., 2020). Citrus papain-like cysteine proteases (PLCPs) are key targets of SDE1, and the SDE1–PLCPs interaction inhibits the protease activity to reduce plant defence responses (Clark et al., 2018). Recently, Pang et al. (2020) found that SDE15 (CLIBASIA_04025) targets the citrus-susceptibility gene ACCELERATED CELL DEATH 2 (CsACD2) to suppress plant immunity and promote the multiplication of CLas (Pang et al., 2020). Besides classical secretory proteins (with a signal peptide), nonclassical secretory proteins (without a signal peptide) also play important roles in CLas pathogenicity. LasBCP (CLIBASIA_RS00445) encodes a peroxiredoxin that reduces the oxidative damage to citrus leaves and accumulates callose in N. benthamiana (Jain et al., 2018, 2019). Recently, 10 CLas nonclassical secretory proteins were identified as cell death suppressors that induce the expression of PR genes to suppress the early hypersensitive response (HR) defence and facilitate colonization (Du et al., 2021).

Prophages are important components of the mobile and integrative genetic elements (MIGEs) that have key roles in bacterial evolution, cell defence, and environmental adaptation (Boyd, 2012; Hendrix, 2002; Ebner & Gtz, 2019). With the wide application of whole-genome sequencing technology, the genomes of over 30 CLas strains have been determined. Three types of prophages (Type 1, Type 2, and Type 3, represented by SC1, SC2, and P-JXGC-3, respectively) have been identified in CLas (Zhang et al., 2011; Zheng et al., 2018). CLas prophages encode multiple genes that may be involved in the adaptability and pathogenicity of CLas (Zhang et al., 2011). The expression of the holin (SC1_gp110) and predicted endolysins (SC1_gp035) of prophage late genes inhibited the growth and feeding by insect vectors of Escherichia coli and Liberibacter crescens, suggesting that the two prophage genes may participate in limiting the host range and ability to be cultured (Fleites et al., 2014). Transgenic citrus plants expressing LasP235 (SC1_gp235) showed HLB-like symptoms, demonstrating that this gene in the prophage region may be a symptom determinant of CLas (Hao et al., 2019). Transient expression of SC2_gp095, a putative nonclassical secretory protein gene, in N. benthamiana inhibited the reactive oxygen-mediated host defence response (Jain et al., 2015).

At present, little is known about the predicted secretory proteins from CLas prophage regions and their functions. In this study, we provide a genome-wide identification of secretory proteins from representative CLas prophage types. A nonclassical secretory protein AGH17470 was identified by bioinformatic prediction coupled with a laboratory-based E. coli alkaline phosphatase (PhoA) assay. AGH17470 induced systemic cell necrosis and dwarfing in N. benthamiana. Overexpression of AGH17470 triggered the accumulation of local reactive oxygen species (ROS), up-regulated pathogenesis-related (PR) family genes, and prompted salicylic acid (SA) synthesis in N. benthamiana and citrus plants. Our results reveal that AGH17470, a nonclassical effector in the prophage region, may serve as a betrayed virulence factor and thereby provides clues to the plant defence response against CLas.

2 | RESULTS

2.1 | AGH17470 is a nonclassical secretory protein in the prophage region of CLas

To predict the classical and nonclassical secretory proteins of prophage regions, genomes of three prophages, p-gxpsy-1, p-gxpsy-2, and p-JXGC-3, were subjected to bioinformatic analysis (Lin et al., 2013; Zheng et al., 2018). An in silico analysis showed that 19 unique classical and nonclassical secretory proteins were predicted from the three prophage genomes. The same secretory proteins predicted from different prophage genomes are shown in the same colour in Table S1. AGH17470, which shares 99.7% amino acid similarity with SC2_gp095 (Jain et al., 2015), was not selected for further study. Of the remaining 18 proteins, 14 were nonclassical secretory proteins and four were classical secretory proteins. Based on sequence conservation of putative secretory proteins in three prophage genomes (Figure S1a) and the thresholds of nonclassical secretory protein (secP score >0.5), a hypothetical protein AGH17470 with the highest score among the conserved proteins was further analysed in this study.

Because SignalIP 3.0 predicted the most likely cleavage site (1–15 amino acids) of AGH17470 and the first and last 50 amino acids may contribute to secretion of nonclassical secretory proteins (Niu et al., 2021), seven truncated segments (amino acids 1–14, 1–50, 1–166, 167–215, 51–215, 15–215, and 1–215) were subjected to an alkaline phosphatase (PhoA) fusion assay for determining the key sequence involved in secretion. The results demonstrated that the first 50 and the last 50 amino acid sequences of AGH17470 are crucial for its secretion (Figure 1).

We next examined the expression pattern of AGH17470 in CLas-infected citrus and psyllids. Reverse transcription-quantitative PCR (RT-qPCR) analysis showed that the gene expression level of AGH17470 in CLas-infected sweet orange was significantly higher (c.37-fold) than that in psyllids (Figure S1b), implying its biological significance in planta.

2.2 | Transient expression of AGH17470 triggers cell death in N. benthamiana

To explore the potential role of AGH17470 in CLas virulence, AGH17470 was transiently expressed in N. benthamiana by using a potato virus X (PVX) vector, and the resulting phenotypes were observed. The pro-apoptotic mouse BCL2-associated X protein
BAX) that can trigger HR-based cell death was used as the positive control (Lacomme & Santa Cruz, 1999). As shown in Figure 2a, AGH17470 induced cell death symptoms that were similar to those of BAX-triggered HR-based cell death in N. benthamiana, whereas similar symptoms were not observed in N. benthamiana infiltrated with PVX-green fluorescent protein (GFP) and buffer. Ion leakage caused by necrosis was detected, and it was higher than PVX-GFP at 7 days postinfiltration (dpi) (Figure 2b). Transient expression of His-AGH17470 induced the same symptoms as AGH17470 (Figure 2c).

To determine the key segment involved in the induction of necrosis, transient expression of four truncated proteins of AGH17470 showed that only the full-length protein of AGH17470 induced necrosis and deposition of H2O2 (Figure 2d). The expression of the truncated protein was confirmed by western blotting, demonstrating that the cell death symptoms were indeed triggered by AGH17470 (Figure 2e). In addition to cell death at the infiltrated site, AGH17470 caused severe interveinal necrosis and deformity of the systemic leaves as well as dwarfing of the entire plant (Figure S2a,b).

RT-qPCR analysis revealed that the PVX accumulation level of PVX-AGH17470 had no significant difference to that of PVX-GFP in N. benthamiana (Figure S2c), indicating that the appearance of systemic necrosis symptoms was not related to virus accumulation. Collectively, these results demonstrate that AGH17470 triggers HR-based cell death in N. benthamiana.

To investigate the molecular evidence for triggering HR in N. benthamiana, the expression levels of an HR marker gene, Harpin-induced 1 (HIN1), and pathogenesis-related (PR) genes were evaluated by RT-qPCR. The results showed that the expression level of NbHIN1 was significantly elevated after infiltration, reaching a peak at 7 dpi (Figure 3a). The expression levels of five PR genes, NbPR1, NbPR2, NbPR3, NbPR4, and NbPR5, were significantly up-regulated after transient expression of AGH17470 (Figure 3b–f). Because NbPR1, NbPR2, and NbPR5 are defence response-related genes of the SA pathway, we further examined the expression of NbNPR1, NbTGA, and NbEDS1, the upstream regulators of the SA signalling pathway (Vleesschauwer et al., 2008; Vlot et al., 2009). The results showed that NbNPR1, NbTGA, and NbEDS1 were highly expressed in transient overexpression plants (Figure 3g–i).

To determine the response of the citrus host to AGH17470, we constructed a plant expression vector pAGH17470 in which AGH17470
is driven by the cauliflower mosaic virus 35S (CaMV 35S) promoter, and further generated AGH17470-transgenic Wanjincheng (Citrus sinensis) via Agrobacterium-mediated epicotyl transformation. AGH17470-transgenic shoots developed chlorotic and yellowing symptoms, while wild-type shoots remained vigorous (Figure 4a). The transgenic lines 1–5 were confirmed by reverse transcription
PCR (RT-PCR) and β-glucuronidase (GUS) histochemical staining (Figure 4b,c). Relative expression analysis showed that line 4 had the highest expression level of AGH17470, followed by line 3 and line 5, and line 2 had the lowest relative expression level (Figure S4). Leaf RNA was extracted to detect the expression of SA-mediated signal transduction-related genes. The RT-qPCR results showed that CsNPR1, CsTGA, CsPR1, CsPR2, CsPR3, and CsPR5 genes were significantly up-regulated in all five transgenic lines (Figure 4d).

2.5 | Overexpression of AGH17470 enhances accumulation of free SA in N. benthamiana and citrus

As the expression of SA-mediated signal transduction genes was significantly up-regulated, we further examined the free SA content in the shoots of the AGH17470-transgenic citrus and the leaves of N. benthamiana and citrus transiently expressing AGH17470 by high-performance liquid chromatography (HPLC). The data indicated that free SA significantly accumulated during both transient and stable expression of AGH17470 in plants (shoots) (Figure 5).

2.6 | Transient expression of AGH17470 in sweet orange enhances resistance to citrus bacterial canker

Xanthomonas citri subsp. citri (Xcc) is a gram-negative bacterium responsible for citrus bacterial canker, which is another devastating citrus bacterial disease worldwide (Omar et al., 2018; Schaad et al., 2005). To assess the citrus bacterial canker resistance of citrus
plants expressing AGH17470, AGH17470 was transiently expressed in citrus leaves and in vitro assays were performed via fine needle inoculation of Xcc. Through RT-PCR, we detected that a c.670 bp fragment of AGH17470 was present in transient expression citrus leaves (lanes 1–5) but not in empty vector control leaves (lanes 6–10) (Figure 6a).
transient expression of AGH17470 and thus AGH17470 lesions are reduced by transient expression of AGH17470 to promote the biosynthesis of SA, and enhance plant disease resistance in plants.

Pathogen recognition by the plant immune system leads to defence responses that are often accompanied by an HR (Jones & Dangl, 2006; Nimchuk et al., 2003). To counteract the HR, a strong immune response that is accompanied by localized cell death to prevent the spread of a pathogen, bacteria, fungi, and oomycetes often secrete a set of effectors that contribute to cell death suppression and thereby effect successful infection (Coll et al., 2011). In CLas, most of the reported secretory proteins play roles in inhibiting the HR to promote bacterial infection (Du et al., 2021; Pang et al., 2020; Zhang et al., 2019, 2020), and few secretory proteins can directly stimulate the HR in plants. Compared with chromosomal genes CLIBASIA_05315 and CLIBASIA_00460 (Liu et al., 2019; Marco et al., 2016), the symptoms induced by AGH17470 were more severe. To our knowledge, this is the first secretory protein from prophage regions in CLas that has been identified as an HR elicitor and induces strong hypersensitive cell death.

The HR, as a plant defence response, usually induces rapid ion channel opening, a burst of ROS, and the accumulation of SA, jasmonic acid, and other signal molecules, thus activating the systemic acquired resistance (SAR) of plants (Hahn, 1996; Meenakshi & Singh, 2013; Pontier et al., 1998; Verberne et al., 2000). Overexpression of AGH17470 induced a high level of electrolyte leakage and the accumulation of ROS. Peroxidase SC2_gp095, another prophage-encoded gene, scavenges ROS (Jain et al., 2015), and thus the two secretory proteins might function cooperatively. It would be interesting to explore the potential interactions between CLas secretory proteins inducing or inhibiting the HR. High expression of PR proteins is an important marker of SAR (Balint-Kurti, 2019; Coll et al., 2011). The overexpression of AGH17470 in N. benthamiana and citrus plants elevated the expression of PR genes and SA-signalling pathway genes, and thus might trigger a plant defence response. This is consistent with the up-regulation of SA signalling pathway-related genes on CLas infection (Kim et al., 2009; Zhong et al., 2015). It should be noted that 10 CLas secretory proteins identified as cell death suppressors can induce the up-regulation of PR1, PR2, and PR5 (Du et al., 2021). Although the mechanism of how the 10 effectors enhanced expression of PR1, PR2, and PR5 is not clear, it may represent a novel virulence

So far, progress has been made towards elucidating the molecular mechanism of CLas pathogenicity depending on characterization of the bacterial secretory proteins and their targets. However, most of the identified secretory proteins were from the CLas chromosomal regions (Du et al., 2021; Liu et al., 2019; Pitino et al., 2016, 2018; Shi et al., 2019; Zhang et al., 2019, 2020), and the functions of secretory proteins from prophage regions remain largely unknown. In this study, we identified a nonclassical secretory protein AGH17470 from the CLas prophage regions. Transient expression of AGH17470 caused cell death and ROS accumulation in N. benthamiana. Moreover, AGH17470 was able to induce the expression of PR genes and SA pathway-related genes, promote the biosynthesis of SA, and enhance plant disease resistance in plants.

FIGURE 4 Salicylic acid (SA) signalling pathway genes were highly expressed in the AGH17470-transgenic citrus. (a) The phenotypes of AGH17470-transgenic citrus shoots. The wild-type citrus plants were also generated as negative control images 1–5. Images 6–10 are positive transgenic lines of 1–5. (b) Confirmation of the AGH17470-transgenic citrus by reverse transcription-PCR. M, DL2000 marker; +, positive recombinant plasmid control; −, negative control of empty vector. Lanes 1–5 lanes show wild-type citrus lines 1–5; lanes 6–10 show AGH17470-transgenic citrus lines 1–5. (c) Validation of the transgenic lines by β-glucuronidase (GUS) histochemical staining. Tubes 1–5 are negative controls and tubes 6–10 are positive transgenic lines of 1–5. (d) Reverse transcription-quantitative PCR analysis of pathogenesis-related (PR) genes and SA signalling pathway-related genes in different transgenic lines. The CsGAPC1 gene was used as an endogenous control. Error bars indicate standard errors of means, and the letters represent significant differences among samples by one-way analysis of variance followed by least significant difference post hoc tests at a p = 0.05. All experiments were repeated three times.

GUS histochemical staining revealed a blue colour on the periphery of the leaf discs that did not appear in the wild type (WT) (Figure 6b). Less severe symptoms (smaller spongiform lesion) were observed in the transient expression of AGH17470 leaves compared with the control leaves at 7 dpi (Figure 6c). Smaller canker lesion sizes and fewer Xcc colonies were found in the transient expression of AGH17470 citrus leaves (Figure 6d,e). Together, these findings suggest the Xcc lesions are reduced by transient expression of AGH17470 and thus transient expression of AGH17470 enhances Xcc resistance.

FIGURE 5 Accumulation of free salicylic acid (SA) in transient expression of AGH17470 and AGH17470-transgenic citrus plants. PVX-AGH17470 was used for the transient expression of AGH17470 in Nicotiana benthamiana; pLGN-AGH17470 was used for transient expression of AGH17470 in citrus. The transient expression leaves of N. benthamiana and citrus were collected at 5 days postinoculation, and transgenic citrus were collected 2 months after infiltration. Four biological replicates were performed, and the error bars indicate standard errors (**p < 0.01, *p < 0.05, t test).
characteristic and reflect the complexity of functions of CLas effectors.

Transient expression of AGH17470 in citrus plants can enhance the resistance to citrus bacterial canker, which is consistent with the findings that triggering the SAR response protects citrus plants from important citrus fungi and bacterial diseases such as HLB, citrus canker, and black spot (Dutt et al., 2016; Ward et al., 1991; Zhang et al., 2010). Further identification of the interaction factors in plant hosts will address the question of how AGH17470 induces the HR and enhances the expression of PR and SA-signalling pathway genes. Although significant progress has been made in understanding the interactions between effector proteins and host targets, the interaction regulation network of CLas secretory proteins remains elusive. Because HLB usually has a long latent period before producing visible symptoms, we speculate that there are other secretory proteins that might function coordinately with AGH17470 to regulate the plant defence response during different stages of CLas infection.
In conclusion, the present study identified a nonclassical secretory protein in the CLas prophage regions that could cause hypersensitive cell death after transient expression in N. benthamiana. Overexpression of AGH17470 in citrus plants up-regulated the transcription of PR genes and SA-signalling pathway genes, and promoted the accumulation of SA. This in turn induced resistance to Xcc, yielding a reduced disease severity phenotype. The present findings have increased our understanding of prophage-encoded secretory protein genes and have provided clues to the plant defence response against CLas.

4 | EXPERIMENTAL PROCEDURES

4.1 | Plants, microbial strains, and growth conditions

N. benthamiana used in this study was grown in a greenhouse at 25°C with 16 h of light and 8 h of darkness. The AGH17140 transgenic shoots were grown in light incubator at 28°C under a 16 h/8 h light/dark cycle. The C. sinensis ‘Wanjincheng’ plants were grown in a greenhouse with the temperature maintained at 28°C. E. coli was cultured at 37°C and Agrobacterium tumefaciens was cultured at 28°C. The bacterial culture was stored in an ultralow temperature refrigerator at −80°C, with a final concentration of 20% glycerol. The final concentrations of antibiotics used in the Luria Bertani (LB) liquid and solid media for culturing the recombinant strains were kanamycin 50 μg/ml and rifampicin 20 μg/ml.

4.2 | Bioinformatic identification of the secretory proteins in the prophage region of CLas

The prophage sequences used for identification and analysis were extracted from the genomes of CLas gypsy strain (GenBank accession GCA_000346595.1) from Guangxi and JXGC strain (GCA_002216815.1) from Jiangxi, China (Lin et al., 2013; Zheng et al., 2018). The signal peptide prediction software SignalP (versions 3.0, 4.0, and 5.0), Phobius, and TMHMM 2.0 were used to exclude proteins containing transmembrane domains (Almagro Armenteros et al., 2019; Bendtsen et al., 2004; Käll et al., 2007; Krogh et al., 2000; Petersen et al., 2011). SecretomeP 2.0 software was used to predict nonclassical secretory protein (ncSecPs) (Bendtsen et al., 2005).

4.3 | Alkaline phosphatase assay

The full-length or specific peptide-coding sequence of AGH17470 was amplified from the template DNA using specific primers (Table S2), and the stop codons were removed to prevent premature termination of transcription. Then, the PCR products were double digested with restriction enzymes and cloned into the pET-mphoA vector (Liu et al., 2019). The resultant recombinant vector was transformed into E. coli BL21. The transformed BL21 strain was spotted on indicator medium (LB solid medium containing 90 μg/ml 5-bromo-4-chloro-3-indolyl phosphate [BCIP], 100 mM isopropyl-β-D-thiogalactopyranoside [IPTG], and 200 μl of 1 M Na2HPO4 coated on each indicator plate). Blue colonies were considered to have alkaline phosphatase (PhoA) activity, indicating that the inserted sequence could guide the secretion of PhoA to the outside of the cell, while white colonies indicated a lack of PhoA activity.

4.4 | Nucleic acid extraction and RT-qPCR analysis

The expression levels of genes such as AGH17470, NbHIN1, and NbPR were analysed by RT-qPCR. The sweet orange leaf veins (Citrus sinensis), psyllids, N. benthamiana leaves after transient expression of AGH17470 protein, and positive transgenic AGH17470 citrus plants were quickly frozen in liquid nitrogen and ground into powder. The total RNA was extracted using TRIZol reagent (Tiangen) according to the manufacturer’s instructions. A PrimeScript RT Reagent Kit with gDNA Eraser (Takara) was used for DNA removal and cDNA synthesis. The CLas-infected citrus leaf veins and psyllids from citrus groves in Ganzhou city, Jiangxi province were examined for infection by RT-qPCR assay. The primers listed in Table S2 and NovoStart SYBR qPCR SuperMix Plus Kit (Novoprotein) were used in qPCR amplification. The LasgynA gene (GenBank no. CP001677.5), N. benthamiana elongation factor 1-α (EF1-α) gene homologous sequence (XM_016613715.1), and the C. sinensis glyceraldehyde-3-phosphate dehydrogenase GAPC1 (XM_006476919.3) were used as internal reference genes. The qPCR was performed with 20 μl of the reaction mixture containing 10 μl of 2 × NovoStart SYBR qPCR SuperMix (Novoprotein), 0.5 μl of forward and reverse primers (10 μM), 2 μl of cDNA (50 ng/μl), and 7 l μl of RNase-free double-distilled water. The cycle programme was as follows: initial denaturation at 95°C for 30 s and then 40 cycles of amplification (95°C for 10 s, 58°C for 30 s, and 72°C for 30 s). Each sample was used for three technical replicates and at least three independent biological replicates were performed. The 2ΔΔCT method (Livak & Schmittgen, 2001) was used for expression calculation and Student’s t test (SPSS 10.0) was used for statistical analysis of all data.

4.5 | Agro-infiltration assay in N. benthamiana

The full-length sequences of AGH17470 were amplified and inserted into the binary vector potato virus X (PVX) digested by ClaI/Sall (Liu et al., 2019). PVX-GFP and PVX-BAX were used as negative and positive controls, respectively, and were transformed into A. tumefaciens GV3101 together with the recombinant vector PVX-AGH17470. The A. tumefaciens culture was centrifuged and resuspended with infiltration buffer (10 mM 2-(N-morpholino)ethanesulfonic acid [MES], 10 mM MgCl2, and 10 μM acetosyringone) to OD600 = 0.6. After the suspension had been kept at room temperature and in the dark...
for 3 h, it was infiltrated into four to six leaves of *N. benthamiana* using sterile syringes. The symptoms were observed and photographed. Then, we amplified His-AGH17470, His-GFP, and four deletion mutant fragments with a His tag containing AGH17470N1 (amino acid residues 101–216), AGH17470N2 (residues 51–216), AGH17470C1 (residues 1–166), and AGH17470C2 (residues 1–115), and constructed the recombinant vectors by the same method and repeated the transient expression in *N. benthamiana*.

### 4.6 Electrolyte leakage measurement

To determine the electrolyte leakage associated with the HR induced by AGH17470, five leaf discs, each about 5 mm in diameter, of the *Agrobacterium*-infiltrated *N. benthamiana* were collected at 3, 5, 7, 9, and 11 dpi. The leaf discs were immersed in 10 ml of double-distilled water and shaken in a shaker (160 rpm) at 28°C for 1 h, and a Mettler-Toledo FE30 conductivity meter was used to determine the conductivity of the solution.

### 4.7 3,3’-diaminobenzidine staining

The *N. benthamiana* leaves were collected at 5 dpi and soaked in 3,3’-diaminobenzidine (DAB) solution (1 mg/ml DAB, 1:2000 Tween 20, and 10 mM Na$_2$HPO$_4$), then vacuumed three to five times until the DAB solution infiltrated the plant leaves. The leaves with stain solution were placed on a 60–70 rpm plate shaker and shaken at room temperature for 8 h. To prevent the DAB from being decomposed by light, the leaves were placed in the dark. Finally, the leaves were decolourized with anhydrous ethanol until the green colour faded.

### 4.8 Subcellular localization of AGH17470 in plant cells

The coding sequence of AGH17470 (with the stop codon deleted) was amplified with the corresponding primers pCHF3-AGH17470-GFP-F/ pCHF3-AGH17470-GFP-R (Table S2) and then digested with a single enzyme BamH1 and inserted into the binary vector pCHF3-GFP (Li et al., 2021). Sequencing was used to ensure the correct ligation to create the recombinant vector pCHF3-AGH17470-GFP. The obtained vector was transformed into *A. tumefaciens* C58C1. *A. tumefaciens* infiltration was conducted on *N. benthamiana* leaves at the four- to six-leaf stage. The infiltrated leaves were collected at 60 hpi and an FV3000 confocal microscope (Olympus) was used to observe the GFP fluorescence.

### 4.9 Western blot analysis

The total protein was extracted from the infiltrated transient expression leaves according to the manufacturer’s instructions (Biotime). Subsequently, the total protein was separated by 12% SDS-PAGE. The protein samples were transferred to a polyvinylidene difluoride (PVDF) membrane. Anti-His tag antibody was used at a dilution of 1:3000 and incubated with the membrane overnight. Then, the membrane was transferred to a 5% skim milk solution containing goat anti-mouse IgG coupled with horseradish peroxidase (HRP). After incubation at room temperature for 3 h, the blot was incubated in Super ECL Western Blotting Reagent (American Everbright Company) to detect chemiluminescence. Western blot analysis was also conducted referring to the previous steps. The primary and secondary antibodies were anti-GFP mAb and HRP-conjugated goat anti-rabbit IgG for subcellular localization. The experiments were repeated three times.

### 4.10 Overexpression vector construction and plant transformation

The full-length AGH17470 was PCR amplified using pLGN-AGH17470F/R (Table S2) and cloned into pLGNe to yield a recombinant plasmid (Li et al., 2020). *Agrobacterium*-mediated transformation of epicotyl segments of *C. sinensis ‘Wanjincheng’* was performed using *A. tumefaciens* EHA105 as previously described (He et al., 2019; Li et al., 2020). The transgenic AGH17470 citrus plants were confirmed by RT-PCR using previous amplification primers, and GUS activity was assessed through histochemical analysis. Subsequently, the leaves were collected for RT-qPCR analysis and sent to Suzhou Grace Biotechnology Co., Ltd for determination of free SA content through HPLC.

### 4.11 Citrus transient expression and Xcc infiltration assays

The EHA105 single clone of pLGN-AGH17470 was cultured in LB liquid medium containing 20 μM acetosyringone (AS). The *A. tumefaciens* pLGN-AGH17470 cells were resuspended in infiltration buffer (0.446% Murashige & Skoog salts, 0.4% sucrose, 20 mM MES, 10 mM MgCl$_2$, and 150 μM AS). The OD$_{600}$ was adjusted to 0.8, and the suspension was maintained in the dark for 1 h before infiltration. Syringes were used to inject the leaves of Wanjincheng plants (Zhao et al., 2021). Leaves infiltrated with the empty vector were used as controls. The leaves were collected at 1, 3, 5, 7, and 9 dpi for RT-PCR and the infiltrated leaf discs on the fifth day were harvested for GUS histochemical staining and then decolourised with 70% ethanol (He et al., 2019; Sendín et al., 2017). At the same time, the leaves on the fifth day were collected for determination of free SA content.

The leaves after 1 day of agro-infiltration were inoculated with Xcc to analyse the susceptibility. Before inoculating with Xcc, the leaves were surface-sterilized with 75% ethanol and inoculated in an asepsis work table. Pins (0.5 mm) were used to artificially create a wound, and 1 μl of each Xcc suspension (OD$_{600}$ <0.8)
was subsequently inoculated. After inoculation, the leaves were cultured in sterile dishes and petioles were moisturized. Canker symptoms were imaged at 7 dpi and ImageJ software (National Institutes of Health) was used to analyse the diseased spots. The leaves of six spots were collected and fully ground with double-distilled water. After serial dilution, the liquids were spread on LB solid plates and cultured at 28°C for 3 days. Then, specific primers Xcc-F/R were used to identify the colonies of Xcc and the colony-forming units of six spots were counted. The experiment comprised three biological replicates.

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CONFLICT OF INTEREST
All authors declare they have no conflict of interest.

DATA AVAILABILITY STATEMENT
The data used to support the findings of this study are available from the corresponding author upon request.

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