"*Candidatus Liberibacter asiaticus*" Secretes Nonclassically Secreted Proteins That Suppress Host Hypersensitive Cell Death and Induce Expression of Plant Pathogenesis-Related Proteins

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ABSTRACT Although emerging evidence indicates that bacteria extracellularly export many cytoplasmic proteins referred to as nonclassically secreted proteins (ncSecPs) for their own benefit, the mechanisms and functional significance of the ncSecPs in the extracellular milieu remain elusive. "*Candidatus Liberibacter asiaticus*" (CLas) is a fastidious Gram-negative bacterium that causes Huanglongbing (HLB), the most devastating citrus disease globally. In this study, using the SecretomeP program coupled with an *Escherichia coli* alkaline phosphatase assay, we identified 27 ncSecPs from the CLas genome. Further, we demonstrated that 10 of these exhibited significantly higher levels of gene expression in citrus than in psyllid hosts and particularly suppressed hypersensitive response (HR)-based cell death and H₂O₂ overaccumulation in *Nicotiana benthamiana*, indicating their opposing effects on early plant defenses. However, these proteins also dramatically enhanced the gene expression of pathogenesis-related 1 protein (PR-1), PR-2, and PR-5, essential components of plant defense mechanisms. Additional experiments disclosed that the increased expression of these PR genes, in particular PR-1 and PR-5, could negatively regulate HR-based cell death development and H₂O₂ accumulation. Remarkably, CLas infection clearly induced gene expression of PR-1, PR-2, and PR-5 in both HLB-tolerant and HLB-susceptible species of citrus plants. Taking together these data, we hypothesized that CLas has evolved an arsenal of ncSecPs that function cooperatively to overwhelm the early plant defenses by inducing host PR genes.

IMPORTANCE In this study, we present a combined computational and experimental methodology that allows a rapid and efficient identification of the ncSecPs from bacteria, in particular unculturable bacteria like CLas. Meanwhile, the study determined that a number of CLas ncSecPs suppressed HR-based cell death and thus indicated a novel role for the bacterial ncSecPs in the extracellular milieu. More importantly, these ncSecPs were found to suppress cell death presumably by utilizing host PR proteins. The data overall provide a novel clue to understand the CLas pathogenesis and also suggest a new way by which phytopathogens manipulate host cellular machinery to establish infection.

KEYWORDS "*Candidatus Liberibacter asiaticus*," nonclassically secreted protein, hypersensitive response, cell death suppression, pathogenesis-related protein

Bacteria often secrete suites of proteins that contribute to environmental functioning and bacterial virulence (1–3). The secreted proteins generally possess appropriate signal peptides (SPs) or translocation signals that enable targeting of a variety of

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conventional secretion pathways, including type I to VII secretion systems (T1SS to T7SS), in addition to Sec- and Tat-dependent translocation pathways (4–9). Interestingly, many cytosolic proteins (CPs) are also exported to the extracellular milieu to aid bacterial growth despite their lack of recognized SPs or translocation signals (10–14) and have thus been referred to as nonclassically secreted proteins (ncSecPs) (15, 16).

ncSecPs have been repeatedly documented in recent decades, but it is still unclear how they are released into extracellular environments. Bacterial cells in the stationary growth phase or with increased autolytic activity or cell fragility have been observed to release more CPs to the supernatant (14, 17–19), indicating a contribution of cell lysis to nonclassical secretion. However, other observations indicate that the release of CPs is not simply due to cell lysis but is also governed by specific mechanisms, as in outer membrane vesicles (OMVs) (20–22). For example, a significant number of CPs have been observed to associate with OMVs in both Gram-negative and Gram-positive bacteria (23, 24), suggesting the potential for OMVs to mediate nonclassical secretion. In addition, the SecA2 secretion system of mycobacteria is also assumed to participate in CP export since deletion of *secA2* in *Mycobacterium tuberculosis* dramatically reduces the abundance of superoxide dismutase (SodA) and catalase-peroxidase (KatG), two cytoplasmic proteins without secretion signals, in culture supernatants (25).

Although no general mechanism for nonclassical secretion is known, *in silico* analysis of known bacterial ncSecPs have shown that these proteins harbor distinctive amino acid compositions, secondary structures, and disordered regions, indicating that nonclassical secretion is governed by protein structure (15). The critical role of specific amino acids and domains in nonclassical secretion has been well exemplified by enolase, a known bacterial ncSecP (26–28). In addition, a recent study indicated that the typical cytoplasmic protein RDPE (D-psicose 3-epimerase of *Ruminococcus* sp. 5_1_39BFAA) in *Bacillus subtilis* is exported extracellularly only when properly folded and arranged as a tetrameric complex, providing further evidence that the higher-order structure of proteins is involved in nonclassical secretion (29).

“*Candidatus Liberibacter asiaticus*” (CLas) is a Gram-negative alphaproteobacterium that causes Huanglongbing (HLB; also known as citrus greening), which is the most devastating citrus disease in the world (30–34). CLas is a phloem-limited bacterial pathogen that is naturally transmitted between citrus plants by phloem-feeding insects referred to as Asian citrus psyllids (ACPs). After colonizing phloem tissues, CLas gradually induces disease symptoms, including leaf mottling, discolored fruits, and aborted seeds, ultimately leading to the death of the entire plant (31, 32).

CLas remains unculturable in artificial media. However, a highly reduced CLas genome (~1.2 Mb) that encodes only 1,136 putative proteins was recovered using metagenomics in 2009 (35). Genome analysis indicated that CLas lacks T3SS, T4SS, and T6SS, which are typical systems utilized by Gram-negative bacteria to secrete proteins (also termed effectors) (36, 37). However, CLas possesses the Sec-dependent secretion pathway (35, 38). Several Sec-dependent secretory proteins have been identified (38, 39). Further, investigation of their transcriptional profiles or biological functions have indicated that several Sec secretory proteins, including CLIBASIA_05315, CLIBASIA_03875, CLIBASIA_00460, and CLIBASIA_04025, are involved in host plant infections (39–45). Two CLas-encoded proteins (CLIBASIA_RS00445 and SC2_gp095) were also identified as nonclassical secretory proteins and were shown to suppress plant defense signaling (46–48), indicating that CLas has evolved a nonclassical secretion mechanism to secrete virulence proteins for its own benefit.

In this study, we report the genome-wide identification of CLas ncSecPs using the SecretomeP software package (15) coupled with a laboratory-based *Escherichia coli* alkaline phosphatase (PhoA) assay (41). A total of 27 CLas-encoded proteins were identified as being exported via nonclassical pathways. We further show that 10 of these suppressed hypersensitive response (HR)-based cell death in plants, presumably by inducing gene expression of pathogenesis-related (PR) proteins that are key components of plant

TABLE 1 Selected putative ncSecPs of CLas subjected to PhoA assays^a

Protein	Gene locus			Annotation	PhoA assay ^b
	psy62	Gxpsy	Ishi-1		
ncSecP1	CLIBASIA_04710	WSI_04540	CGUJ_04710	Hypothetical protein	*
ncSecP2	CLIBASIA_00845	WSI_00760	CGUJ_00845	Hypothetical protein	***
ncSecP3	CLIBASIA_01580	WSI_01490	CGUJ_01580	Hypothetical protein	*
ncSecP4	CLIBASIA_02070	WSI_03320	CGUJ_02070	Hypothetical protein	N
ncSecP5	CLIBASIA_05190	WSI_05045	CGUJ_05190	Hypothetical protein	*
ncSecP6	CLIBASIA_02830	WSI_02570	CGUJ_02830	Hypothetical protein	**
ncSecP7	CLIBASIA_02025	WSI_03365	CGUJ_02025	Hypothetical protein	N
ncSecP8	CLIBASIA_03865	WSI_03715	CGUJ_03865	Hypothetical protein	*
ncSecP9	CLIBASIA_02590	WSI_02805	CGUJ_02590	Hypothetical protein	*
ncSecP10	CLIBASIA_05210	WSI_05065	CGUJ_05210	Hypothetical protein	**
ncSecP11	CLIBASIA_01670	WSI_01580	CGUJ_01670	Hypothetical protein	N
ncSecP12	CLIBASIA_00580	WSI_00495	CGUJ_00580	Hypothetical protein	*
ncSecP13	CLIBASIA_01120	WSI_01035	CGUJ_01120	Hypothetical protein	***
ncSecP14	CLIBASIA_00370	WSI_00280	CGUJ_00370	Hypothetical protein	*
ncSecP15	CLIBASIA_02305	WSI_03090	CGUJ_02305	Hypothetical protein	***
ncSecP16	CLIBASIA_05155	WSI_04985	CGUJ_05155	Hypothetical protein	**
ncSecP17	CLIBASIA_02150	WSI_03240	CGUJ_02150	NADH dehydrogenase	N
ncSecP18	CLIBASIA_00215	WSI_00130	CGUJ_00215	Hypothetical protein	*
ncSecP19	CLIBASIA_04410	WSI_04235	CGUJ_04410	Hypothetical protein	**
ncSecP20	CLIBASIA_04585	WSI_04420	CGUJ_04585	Hypothetical protein	***
ncSecP21	CLIBASIA_03920	WSI_03755	CGUJ_03920	Hypothetical protein	*
ncSecP22	CLIBASIA_05205	WSI_05060	CGUJ_05205	Hypothetical protein	*
ncSecP23	CLIBASIA_02715	WSI_02680	CGUJ_02715	Hypothetical protein	**
ncSecP24	CLIBASIA_04530	WSI_04370	CGUJ_04530	Hypothetical protein	**
ncSecP25	CLIBASIA_01980	WSI_03410	CGUJ_01980	Hypothetical protein	*
ncSecP26	CLIBASIA_03110	WSI_02300	CGUJ_03110	Flp family type IVb pilin	***
ncSecP27	CLIBASIA_01885	WSI_01790	CGUJ_01885	Site-specific tyrosine recombinase	*
ncSecP28	CLIBASIA_04440	WSI_04265	CGUJ_04440	Hypothetical protein	*
ncSecP29	CLIBASIA_00475	WSI_00380	CGUJ_00475	Hypothetical protein	N
ncSecP30	CLIBASIA_04930	WSI_04760	CGUJ_04930	Hypothetical protein	*
ncSecP31	CLIBASIA_00295	WSI_00205	CGUJ_00295	Hypothetical protein	*
ncSecP32	CLIBASIA_02945	WSI_02460	CGUJ_02945	Heat shock protein	**

^aThe gene locus and annotation are based on the complete genomic data available for three CLas strains: psy62 (GenBank no. [NC_012985](#)), gxpsy (GenBank no. [NC_020549](#)), and Ishi-1 (GenBank no. [NZ_AP014595.1](#)).

^bPhoA assays were performed as described in Materials and Methods. Single (*), double (**), and triple asterisks (***) indicate weak, moderate, and strong PhoA activity, respectively. N, no PhoA activity.

innate immune responses (49–51). These results reveal the importance of CLas ncSecPs in citrus plant infections and shed new light on bacterium-plant interactions.

RESULTS

In silico analysis of nonclassically secreted proteins in CLas. The SecretomeP 2.0 software package (15) was used to predict ncSecP from three complete, annotated genome sequences of CLas strains that originated from different regions of the world, including psy62 from Florida, USA (35); gxpsy from Guangxi, China (52); and Ishi-1 from the Okinawa prefecture, Japan (53). After removing redundant genes, totals of 84, 84, and 75 candidate ncSecPs from psy62, gxpsy, and Ishi-1 (see Table S1 in the supplemental material) were recovered, respectively. Cluster analysis of the candidate ncSecPs identified a total of 66 orthologous proteins across the three CLas strains (see Fig. S1 and Table S1). Among these conserved orthologs, we substantially targeted those annotated as a “hypothetical protein” in an effort to identify novel proteins with potential roles in CLas virulence. A total of 32 proteins (Table 1) were thus selected for subsequent experiments.

Experimental verification of candidate nonclassically secreted proteins. The *Escherichia coli*-based PhoA assay has been primarily used to identify SP-bearing proteins within bacteria (38, 54–57). In this study, we used the system to investigate *in silico*-predicted bacterial ncSecPs (Fig. 1a). The validity and reliability of the PhoA assay for determining the bacterial ncSecPs were first examined by using *Thermobifida fusca*

FIG 1 Validation of CLas-encoded ncSecPs with *E. coli*-based alkaline phosphatase (PhoA) assays. (a) Schematic of prokaryotic expression cassettes for PhoA and PhoA fusion proteins. The CLas ncSecPs were inserted into pET-mphoA and fused with *mphoA*, which lacks a native signal peptide (SP) sequence, resulting in pET-ncSecP-mphoA. Empty pET-mphoA was used as a negative control, while pET-phoA containing the full-length *phoA* gene was used as a positive control. T7-P represents the T7 promoter, and T7-Ter indicates the T7 terminator. (b) PhoA assays confirmed the secretion of TfCut, EcCsp, and LasRS00445, which are three known ncSecPs of bacteria. *E. coli* cells expressing PhoA fusions were grown on indicator LB medium supplemented with BCIP (90 μ g/ml) and IPTG (100 mM). Na_2HPO_4 (75 mM) was added to the medium to suppress endogenous phosphatase activity of *E. coli*. Color change was continuously observed up to 36 h after inoculation and photos were taken after 12, 24, and 36 h of incubation. TfCut, EcCsp, and LasRS00445 exhibited strong, medium, and weak secretion, respectively. (c) *In silico*-predicted ncSecPs of CLas were evaluated with PhoA assays. The experiments were performed as described above. Representatives of the CLas proteins with strong (ncSecP42), medium (ncSecP22), weak (ncSecP21), and no (ncSecP9) secretion activities are shown.

Cutinase (TfCut) (58), *E. coli* BL21(DE3) cold shock-like protein (EcCsp) (59), and CLas RS00445 (48), which are all proteins known to be exported via a nonclassical pathway. The cells that expressed the fusion proteins TfCut-mphoA or EcCsp-mphoA (where *mphoA* represents a mature PhoA protein without a native SP) turned dark blue after 12 or 24 h of incubation on indicator Luria-Bertani (LB) agar (Fig. 1b), while the cells expressing RS00445-mphoA turned pale blue after 36 h of incubation. However, the cells harboring pET-mphoA, which were used as a negative control, remained white over the course of 36-h incubations.

The color changes unambiguously demonstrated extracytoplasmic secretion of the fusion proteins, indicating that the PhoA assay could serve as a useful system to identify bacterial ncSecPs.

Based on the above studies, the PhoA assay was employed to verify the *in silico* predicted ncSecPs of CLas. A total of 32 candidate ncSecPs (Table 1) were subjected to the PhoA fusion assay. Of the 32 ncSecPs, 5 failed to be secreted because their mphoA fusion proteins did not result in obvious color changes after 36 h of incubation (Table 1 and Fig. 1c). However, the other 27 ncSecP-mphoA fusion proteins displayed PhoA activity in the *E. coli* cells after between 6 and 36 h of incubation, indicating secretion of these ncSecPs.

Identification of nonclassically secreted proteins that suppress hypersensitive cell death in *Nicotiana benthamiana*. The plant HR is a specialized form of programmed cell death (PCD) that restricts the spread of phytopathogens from the infection site (60, 61). To enable successful infection, the phytopathogens may secrete effector proteins to suppress hypersensitive cell death (2, 62, 63). Identification of the effectors that suppress hypersensitive cell death is therefore a useful step to understand the interactions between phytopathogens and their host plants. Hence, based on the 27 CLas ncSecPs identified above, we first investigated their potential in cell death suppression by means of initially expressing the candidate ncSecP, followed by challenge with a well-known HR PCD inducer, mouse BAX (64). A total of 10 CLas ncSecPs, including ncSecP1, -3, -12, -15, -22, -23, -24, -26, -27, and -28, consistently suppressed BAX-triggered cell death (Fig. 2a and b), whereas the rest showed no ability to suppress cell death (see Fig. S2). Next, the 27 CLas ncSecPs were further screened for their abilities to suppress HR PCD triggered by *Phytophthora infestans* INF1 (65). Using the same strategy outlined above, we identified that, among the 10 ncSecPs that suppressed the BAX-triggered HR-based cell death, six (ncSecP12, -22, -24, -26, -27, and -28) exhibited consistent suppression of INF1-triggered cell death, whereas four (ncSecP1, -3, -15, and -23) contributed to partial suppression (Fig. 2a and b). In addition, the 17 ncSecPs without the capacity to suppress BAX-triggered cell death were also unable to block cell death triggered by INF1 (see Fig. S2).

Hypersensitive cell death is usually accompanied by the dramatic accumulation of hydrogen peroxide (H_2O_2), which is an important reactive oxygen species (ROS) (66). We therefore explored the potential of the 10 ncSecPs that suppressed BAX- and INF1-triggered cell death for H_2O_2 generation using histochemical staining with 3,3'-diaminobenzidine (DAB). All of the 10 ncSecPs significantly inhibited overaccumulation of H_2O_2 triggered by BAX and INF1, consistent with their role in hypersensitive cell death suppression (Fig. 2c).

Taken together, these results showed that 10 of the CLas ncSecPs functioned as cell death suppressors and inhibited BAX- and INF1-triggered HR PCD and H_2O_2 accumulation, implicating a role of these CLas-encoded proteins in subverting early defense responses of plants.

Expression of cell death suppressor genes in citrus plants and psyllid hosts.

Given the role of the aforementioned 10 ncSecPs in HR-based cell death suppression, we further evaluated their transcriptional expression in the citrus plants and insect hosts. Total RNAs from the CLas-infected sweet orange plants and psyllids were analyzed using two-step quantitative real-time reverse transcription-PCR (RT-qPCR). Data analysis showed that RNA transcript levels of the 10 *ncSecP* genes were all higher in citrus plants (ranging from 2.92-fold to >100-fold expression) compared to in psyllids (Fig. 3 and see Table S2), indicating significance of these proteins in citrus plants. Among the tested genes, *ncSecP24* and *ncSecP28* were the two most upregulated ones, which exhibited ~146-fold and ~83-fold higher expression levels in citrus plants, respectively, compared to in psyllids.

Subcellular localization of cell death suppressors in *N. benthamiana*. After entering host cells, the pathogen effectors may be localized to specific subcellular compartments where they are then able to manipulate host molecular functions. To determine the subcellular localizations of the 10 CLas-encoded cell death suppressors in plant cells,

FIG 2 Effects of Clas-encoded ncSecPs on hypersensitive response of *N. benthamiana*. (a) Of the 27 identified ncSecPs, 10 suppressed BAX- and INF1-triggered cell death. *N. benthamiana* leaves were inoculated with agrobacterial cells carrying ncSecPs, followed by a second inoculation 24 h later with agrobacterial cells harboring BAX or INF1 gene. The agrobacterial cells carrying GFP gene were included as negative controls. Cell death symptoms were recorded 5 days after Bax or INF1 infiltration. The suppression activities of the ncSecPs toward BAX- and INF1-triggered cell death were evaluated by using the cell death suppression number/infiltration number (CDS/I) index. The results of three independent experiments were combined as averages. The ncSecPs exhibiting consistent suppression (CDS/I > 90%) or partial suppression (90% ≤ CDS/I ≤ 50%) are shown. Asterisks within columns indicate statistically significant differences based on Student's *t* tests (**, *P* < 0.01). (b) Phenotypes of cell death suppression caused by 10 ncSecPs. Agroinfiltration was performed as described above. Photos were taken 5 days after Bax or INF1 infiltration. (c) ncSecP12 inhibited BAX- and INF1-elicited H₂O₂ overaccumulation. Similar activities were observed for ncSecP1, ncSecP3, ncSecP15, ncSecP22, ncSecP23, ncSecP24, ncSecP26, ncSecP27, and ncSecP28 (data not shown). Agroinfiltration of *N. benthamiana* was carried out as described above, and the leaves were removed 2 days after Bax or INF1 infiltration for histochemical staining with DAB.

FIG 3 Relative gene expression of CLas-encoded cell death suppressors in sweet orange plants and psyllid hosts. RT-qPCR was conducted on total RNA prepared from CLas-infected citrus plants and psyllid hosts, with the CLas DNA gyrase subunit A-encoding gene (*LasgyrA*) used as an internal reference. Three biological replicates (each with three technical replicates) were conducted. The relative transcript abundance of each tested gene in CLas-infected citrus plants was normalized against its expression in CLas-infected psyllids. Bars show averages $\pm$ the standard errors of the means, and asterisks indicate statistically significant differences based on Student's *t* tests (**, $P < 0.01$; *, $P < 0.05$).

their encoding genes were individually fused with green fluorescence protein (GFP) reporter genes (Fig. 4a) and transiently expressed in *N. benthamiana* leaves. The green fluorescence of each GFP fusion protein was visualized by confocal microscopy (Fig. 4b), indicating that ncSecP22-GFP and ncSecP28-GFP were evenly distributed among leaf epidermal cells like free GFP. In contrast, ncSecP1-GFP and ncSecP12-GFP were predominantly localized in the nucleus, and the GFP fusions of ncSecP43, ncSecP15, ncSecP23, ncSecP24, and ncSecP26 were mostly prevalent in the cytoplasm and cytoplasmic membranes. Remarkably, ncSecP24-GFP aggregated into large inclusion bodies that were present in the cytoplasm. Taken together, these data indicate that the 10 CLas cell death suppressors exhibited at least three subcellular localization patterns within plant cells.

Transiently expressed cell death suppressors dramatically enhanced the expression of PR-1, PR-2, and PR-5 in *N. benthamiana*. PR proteins are key components of plant defense mechanisms (49–51) and are often regulated by pathogen effectors (67). To probe the mechanisms associated with the aforementioned cell death suppressors in plant defense, we investigated the transcription of the *PR-1*, *PR-2*, and *PR-5* genes in the *N. benthamiana* leaves that initially transiently expressed each of the 10 CLas ncSecPs, followed by challenge with BAX or INF1.

It is known that Bax, as an HR inducer, enhances gene expression of *PR-1* (64). Likewise, we detected here that the transiently expressed Bax in the *N. benthamiana* leaves resulted in 4.6-fold upregulation of *PR-1* transcription, as well as 1.81- and 6.2-fold increases in *PR-2* and *PR-5* mRNA accumulation (see Fig. S3 in the supplemental material). Similarly, INF1 led to 2.9- and 1.8-fold increases in *PR-1* and *PR-5* transcription (see Fig. S3). Thus, to eliminate the interference of BAX and INF1 on *PR* gene expression, the *N. benthamiana* leaves coexpressing GFP and BAX/INF1 were used as controls during RT-qPCR analysis of the *PR-1*, *PR-2*, and *PR-5* expression in the leaves coexpressing ncSecP and BAX/INF1.

As a result, we discovered that the expression levels of *PR-1*, *PR-2*, and *PR-5* were all elevated in the *N. benthamiana* leaves coexpressing each of the 10 CLas ncSecPs and Bax, compared to those in leaves coexpressing GFP and Bax (Fig. 5). In particular, both *PR-1* and *PR-5* were significantly induced, with increased levels ranging from 5.5-fold (ncSecP22) to 62.16-fold (ncSecP23) and from 24.8-fold (ncSecP27) to 41.3-fold (ncSecP23), respectively. A similar

FIG 4 Subcellular localization of CLas-encoded cell death suppressors in *N. benthamiana*. (a) Expression cassettes for the GFP gene and the fusion gene *ncSecP-GFP*. 35S, CaMV 35S promoter; (Continued on next page)

FIG 5 CLas-encoded cell death suppressors enhanced expression of PR genes in *N. benthamiana*. *N. benthamiana* leaves were inoculated with agrobacterial cells expressing the CLas cell death suppressors or GFP, followed by challenge with Bax or INF1 at 24 hpi. The double-infiltrated patches were collected at 24 h after Bax or INF1 infiltration, and the extracted total RNA was then subjected to RT-qPCR analysis to measure the relative expression levels of PR-1, PR-2, and PR-5. Three biological replicates were used, each with three technical replicates. The *actin* gene of *N. benthamiana* was used as an internal reference. Bars show the standard errors of the mean, and asterisks indicate statistically significant differences based on Student's *t* tests (*, $P < 0.05$; **, $P < 0.01$).

result was observed from *N. benthamiana* coexpressing each of the 10 CLas ncSecPs and INF1, with the leaves coexpressing GFP and INF1 as a control (Fig. 5). Expression levels of PR-1 and PR-5 were dramatically increased ranging from 21.5-fold (ncSecP26) to 275.8-fold (ncSecP23) and from 18.4-fold (ncSecP22) to 128.1-fold (ncSecP26), respectively. Taken together, these data clearly indicated that the 10 CLas cell death suppressors activated transcription of PR-1, PR-2, and PR-5 in *N. benthamiana*.

Given the positive roles of PR proteins in promoting basal resistance and systematic acquired resistance (SAR) (51), the increased expression of PR genes indicates that the 10 ncSecPs suppressed HR-based cell death could prime host resistance after expression in *N. benthamiana*. On the other hand, we questioned if increased expression of PR genes was correlated with cell death suppression.

Transient overexpression of PR-1, PR-2, and PR-5 suppressed hypersensitive cell death in *N. benthamiana*. To address the role of induced PR genes in cell death suppression, PR-1, PR-2, and PR-5 were individually transiently expressed in *N. benthamiana*, followed by challenge with BAX or INF1. As a result, we observed that both PR-1 and PR-5 were able to suppress BAX- and INF1-triggered cell death (see Fig. 7a and b). PR-2 was much less effective in inhibiting cell death triggered by INF1, but almost completely suppressed BAX-triggered cell death (Fig. 6a and b). Consistent with

FIG 4 Legend (Continued)

NOS, nopaline synthase terminator. (b) Fluorescence of ncSecP-GFP fusions in the leaf epidermal cells of *N. benthamiana*. Agrobacterial cells expressing each of the ncSecP-GFP fusions were inoculated into the leaves of *N. benthamiana* grown at 25°C. The GFP fluorescence was viewed by confocal microscopy at 60 hpi, and H2B-dsRed was used as a marker for nuclei. Scale bars, 50 μ m.

FIG 6 Effects of PR genes *PR-1*, *PR-2*, and *PR-5* on hypersensitive response of *N. benthamiana*. (a) Suppression activities of *PR-1*, *PR-2*, and *PR-5* on BAX- and INF1-triggered cell death. *N. benthamiana* leaves were infiltrated with agrobacterial cells carrying *PR-1*, *PR-2*, or *PR-5*, followed by a second infiltration 24 h later with agrobacterial cells harboring the BAX or INF1 gene. Agrobacterial cells carrying the GFP gene were used as negative controls. Cell death symptoms were recorded 5 days after *Bax* or *INF1* infiltration. The suppression activities of the PR proteins were evaluated by using the cell death suppression number/infiltration number (CDS/I) index. The results of three independent experiments were combined as averages. Asterisks within columns indicate statistically significant differences based on Student's *t* tests (*, *P* < 0.05; **, *P* < 0.01). (b) Phenotypes of cell death suppression caused by *PR-1*, *PR-2*, and *PR-5*. Agroinfiltration was performed as described above. Photos were taken 5 days after *Bax* or *INF1* infiltration. (c) *PR-1*, *PR-2*, and *PR-5* inhibited BAX- and INF1-triggered H₂O₂ overaccumulation. Agroinfiltration of *N. benthamiana* was carried out as described above, and the leaves were removed 2 days after infiltration of *Bax* or *INF1* for histochemical staining with DAB.

their roles in cell death suppression, *PR-1* inhibited the overaccumulation of H₂O₂ triggered by both BAX and INF1, while *PR-2* inhibited only BAX-triggered H₂O₂ overaccumulation (Fig. 6c). In addition, *PR-5* inhibited the overaccumulation of H₂O₂ triggered by BAX but only partially reduced INF1-triggered H₂O₂ accumulation (Fig. 6c). Taken

FIG 7 CLas infection enhanced the expression of PR-1, PR-2, and PR-5 genes in citrus plant species. (a) Typical healthy and CLas-infected asymptomatic leaves of Huanglongbing (HLB)-tolerant sour pomelo and HLB-susceptible Jincheng orange. PCR products of 1,160 bp (indicated with an arrow) are shown for the CLas-positive samples. (b) Relative gene expression of *PR-1*, *PR-2*, and *PR-5* in CLas-infected citrus plants. Total RNA of the healthy and CLas-infected asymptomatic leaves were subjected to RT-qPCR analysis, with citrus actin genes used as an internal reference. Three biological replicates were performed, with three technical replicates each. The relative expression of each *PR* gene in CLas-infected asymptomatic leaves was normalized against its transcript abundance in healthy leaves. Bars show the standard errors of the means, and asterisks indicate statistically significant differences based on Student's *t* tests (**, $P < 0.01$; *, $P < 0.05$).

together, these data clearly demonstrate that PR-1, PR-2, and PR-5 confer negative effects on hypersensitive cell death development, with PR-1 being most prominent in this respect.

CLas infection elevated the expression of *PR-1*, *PR-2*, and *PR-5* in citrus plants.

Given that 10 of the CLas-encoded ncSecPs had the ability to enhance transcription of *PR-1*, *PR-2*, and *PR-5* in *N. benthamiana*, we sought to determine whether CLas infection could regulate the expression of their orthologous genes in citrus. To test this possibility, we measured the relative expression levels of *PR-1*, *PR-2*, and *PR-5* in the CLas-

infected but asymptomatic citrus leaves that represent early stage of infection (Fig. 7a). Two citrus species plants, the HLB-tolerant sour pomelo (*Citrus grandis* Osbeck) and the HLB-susceptible Jincheng orange (*Citrus sinensis* Osbeck) (68), were included in the experiments. The results showed that expression levels of *PR-1*, *PR-2*, and *PR-5* were all increased in both of the CLas-infected citrus varieties compared to healthy plants (Fig. 7b). Notably, the expression of *PR-1*, the most prominent cell death suppressor (Fig. 6), was significantly upregulated by as much as ~5.64-fold in sour pomelo, whereas it increased only 2-fold in Jincheng orange (Fig. 7b).

DISCUSSION

The widespread application of proteomics in recent years has resulted in increasing numbers of ncSecPs being identified in both Gram-positive and Gram-negative bacteria through secretome analysis (10–14), indicating that nonclassical protein secretion is a general mechanism employed by bacteria (11, 14, 18, 19). However, the biological significance of the ncSecPs in the extracellular milieu remain largely unknown. In this study, we show that an array of ncSecPs from CLas, a plant-associated Gram-negative bacterium, were able to suppress HR-based cell death in plant, providing a novel role for the bacterial ncSecPs.

It is known that CLas, as a fastidious phloem-limited bacterium, is present at very low titers in host plants (33, 34), thus making it hard to directly identify ncSecPs *in situ* with current proteomics technologies. Here, we employed a combined computational and experimental approach to explore the potential ncSecPs of CLas. Bioinformatics screening first identified 66 candidate ncSecPs that are commonly present in CLas strains. Further, using the *E. coli*-based PhoA assay, 27 of the 32 candidate ncSecPs were verified to be exported out of the cytoplasm, but 5 were not secreted. These suggested that *in silico* prediction resulted in false-positive candidates, or alternatively, that the *E. coli*-PhoA-based fusion assay produced false-negative results. In addition, SC2_gp095 and CLIBASIA_RS00445, two recently reported ncSecPs of CLas (46, 48), were not identified based on bioinformatics screening, indicating false negatives in *in silico* prediction. Despite the limitations, bioinformatics screening coupled with the *E. coli*-based PhoA assays nevertheless provided a useful approach to explore ncSecPs from unculturable bacteria like CLas.

Functional analysis of bacterial ncSecPs in extracellular milieu may lead to new understanding of bacteria and host/environment interaction. Evidence from medically important bacteria suggest that many ncSecPs, like typical secretory proteins, also contribute to virulence or niche adaptation. For example, pathogenic *Staphylococcus aureus* strains are able to generate more ncSecPs than nonpathogenic strains (11, 14), and two of their ncSecPs, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and aldolase (FbaA), have been found to exert cytotoxic effects on host cells, in addition to promoting bacterial adherence to host cells (11). Moreover, it was shown that the GAPDH secreted by many streptococcal species can facilitate host colonization (10, 12), or induce apoptosis in murine macrophages (13). Likewise, 10 of the 27 CLas ncSecPs identified in this study were presumed to be involved in host plant infection since they all suppressed BAX- and INF1-triggered hypersensitive cell death and H₂O₂ accumulation in *N. benthamiana*, while also exhibiting higher mRNA transcriptional levels in citrus plants than in psyllids.

The roles of the 10 ncSecPs in HR-based cell death suppression closely resembled those of the Sec-dependent secretory proteins CLIBASIA_03875, CLIBASIA_04025, and CLIBASIA_04405 the only three cell death suppressors known to date in CLas (39, 42, 45). Nevertheless, numerous Sec-dependent secretory proteins in CLas have been suggested to function in bacterial pathogenicity (39–44, 69–71). Further, SC2_gp095 and CLIBASIA_RS00445, the only two confirmed ncSecPs in CLas, have been experimentally shown to compromise ROS-triggered defenses in *N. tabacum* (46, 48), while the latter also suppresses oxylipin-mediated defenses in citrus plants (47). The present data, combined with those from previous studies, indicated that, despite lacking typical

secretion systems like T3SS, T4SS, and T6SS, CLas employed additional ways, including Sec-dependent and nonclassical secretion, to deliver proteins into citrus hosts to combat plant defense mechanisms and thereby facilitate colonization.

HR represents an early defense mechanism and usually induces SAR accompanied by increases in expression of PR genes, including *PR-1*, *PR-2*, and *PR-5* (72–75). It has been shown that many pathogens have evolved effectors to suppress HR-based cell death, with concurrent suppression of PR gene expression (76–80). Conversely, we identified that 10 CLas ncSecPs functioned as cell death suppressors, but instead enhanced transcription of *PR-1*, *PR-2*, and *PR-5*, in particular *PR-1* and *PR-5*. More interestingly, transient overexpression of these PR genes in *N. benthamiana* suppressed or greatly reduced BAX- and/or INF1-triggered cell death and H₂O₂ accumulation. This is reminiscent of the previous studies in which *PR-1* exhibits high constitutive expression in *Arabidopsis* mutants that suppress avirulent pathogen-induced HR (81, 82). Moreover, the result is also consistent with that from a study wherein PR-1 proteins from both plants and animals, when transgenically expressed in tomato plants, effectively inhibit PCD elicited by the fungal toxin fumonisins B1 (83).

Pathogen effectors are known to suppress HR-based cell death via diverse mechanisms, including attacks of host surveillance systems, inhibition of ROS accumulation, and manipulation of host ubiquitin systems (2, 63, 67, 84). Bioinformatics analysis of the 10 cell death suppressors showed that none of them was a putative transcription factor or regulator (data not shown), suggesting indirect regulation of each ncSecPs on the gene expression of *PR-1*, *PR-2*, and *PR-5*. It is also notable that the 10 cell death suppressors exhibited at least three subcellular localization patterns in *N. benthamiana* cells, indicating that the CLas ncSecPs targeted different host cellular factors to upregulate transcription of *PR-1*, *PR-2*, and *PR-5*. Nevertheless, although the present data cannot address how the CLas-encoded cell death suppressors enhanced expression of *PR-1*, *PR-2*, and *PR-5*, it is clear that the increased expression of the PR genes contributed to cell death suppression, thus representing a novel mechanism by which pathogen effectors overcome host HR.

Omics-based approaches have shown that CLas infection upregulates the expression of *PR-1* in diverse citrus plant varieties, including red tangerine (*C. reticulata*) (85), Mexican lime (*C. aurantifolia*) (86), and sweet orange (*C. sinensis*) (87). In concert with that, RT-qPCR analysis used here indicated that *PR-1*, *PR-2*, and *PR-5* were activated in sour pomelo and Jincheng orange in the early stage of CLas infection. Considering that *PR-1*, *PR-2*, and *PR-5* were involved in the desensitization of HR machinery, the induction of PR genes might represent a mechanism by which CLas bacteria prevent ROS overaccumulation and cell death occurrence during infection of citrus plants. In fact, a recent study has disclosed that transgenically expressed tomato P14a (a member of the *PR-1* family) is confined to the margins of lesions in tobacco plants caused by *Pseudomonas syringae* pv. *tabaci*, thereby restricting lesion expansion and bacterial spread (83). On the other hand, given the positive roles of *PR-1*, *PR-2*, and *PR-5* in plant defenses (49–51), increased PR gene expression would inevitably promote citrus plant defenses against CLas infection. It is well known that CLas can systemically infect all known citrus varieties (67, 88) but manifest with extremely low titers and uneven distributions within the plants (89, 90). Collectively, we proposed that the induced PR proteins served as a double-edged sword for CLas and lead to a trade-off between its survival and effectiveness of population spread: increasing the PR gene expression suppressed the early plant defense HR, thus promoting host colonization, but simultaneously enhanced host resistance, and thereby restricted bacterial reproduction and disperse (Fig. 8). The CLas infection induced expression of *PR-1*, *PR-2*, and *PR-5* in both HLB-tolerant citrus (sour pomelo) and HLB-susceptible citrus (Jincheng orange), but *PR-1* in sour pomelo was most significantly upregulated (~5.64-fold), which might account for tolerance of CLas associated with sour pomelo. However, since *PR-1* was the most prominent cell death suppressor compare with *PR-2* and *PR-5*, it would be

FIG 8 Model for CLas deploying nonclassically secreted proteins (ncSecP) to overwhelm hypersensitive responses in citrus plants. CLas is an intracellular bacterium and extracellularly exports no fewer than 10 ncSecPs that localize to different subcellular localizations but function cooperatively to upregulate gene expression of PR-1, PR-2, and PR-5 via unknown ways. The induced PR proteins subsequently lead to cell death suppression but otherwise contribute to defense against CLas.

interesting to explore if HLB-tolerant sour pomelo was actually more inclined to be colonized by CLas than HLB-susceptible Jincheng orange.

In summary, we demonstrated that CLas bacteria have evolved a group of ncSecPs, and in particular, 10 of them cooperatively function to suppress HR-based cell death. To our knowledge, no HR phenotype has ever been observed when CLas infects citrus plants, indicating that the bacteria are able to mitigate the HR mechanism of citrus plants. We have reported the mature form of the CLas-encoded Sec secretory protein CLIBASIA_03875 that likely contributes to HR suppression by regulating the gene expression of cell death regulators, including cyclic nucleotide-gated channels (CNGC), Bax-inhibitor 1 (BI-1), and WRKY9 (39). More recently, a study shows that the mature protein of another CLas-encoded Sec secretory protein CLIBASIA_04025 acts as a HR suppressor through physically interacting with a red chlorophyll catabolite reductase that represses PCD in plants (42). In this study, 10 of the CLas ncSecPs suppressed hypersensitive cell death presumably by coopting host PR proteins, thereby uncovering a novel strategy used by CLas to colonize citrus plants.

MATERIALS AND METHODS

Bioinformatics analysis. The SecretomeP 2.0 Server (15) was used to predict ncSecPs in the CLas strains psy62 (GenBank no. [NC_012985](#)), gypsy (GenBank no. [NC_020549.1](#)), and Ishi-1 (GenBank no. [NZ_AP014595.1](#)) genomes using default settings for Gram-negative bacteria. Proteins with a score above 0.6 were identified as ncSecP candidates. Orthologs of the candidate ncSecPs across the three strains were then determined using the BLASTClust program by clustering proteins into groups using a coverage threshold of 90% and an amino acid identity threshold of 90%. Extensive manual curation was then performed to ensure the genes exhibited proper annotations.

PhoA assay. PhoA assays were performed as previously described (41) for proteins including TfCut (GenBank no. [CP000088](#)), EcCsp (GenBank no. [CP001509](#)), RS00445 (GenBank no. [CP001677](#)), and the candidate ncSecPs of CLas. The coding sequences of each protein were amplified from respective template DNAs using gene-specific primers (Table 2) and then cloned into pET-mphoA, which harbors *mphoA* (which lacks a native SP-coding sequence), resulting in an in-frame gene fusion between the tested gene and *mphoA*. Constructs were then transformed into chemically competent *E. coli* BL21 cells.

TABLE 2 Plasmid constructs generated in this study

Construct	Primer	Primer sequence (5'–3')
PhoA assay		
pET-ncSecP1-mphoA	phoA-np1F phoA-np1R	AAGGAGATATACATATGGCATATTGGCTTGTA AGGCATTTCTGGAAGCTTCAAAGGAGGATTAGATAG
pET-ncSecP2-mphoA	phoA-np2F phoA-np2R	AAGGAGATATACATATGAAAAACGAGAAAGAAT AGGCATTTCTGGAAGCTTACTTCGATTGGAATAGG
pET-ncSecP3-mphoA	phoA-np3F phoA-np3R	AAGGAGATATACATATGGTCTGGACTGTTGAAAG AGGCATTTCTGGAAGCTTTTCTGAATTTGCCTGAAC
pET-ncSecP4-mphoA	phoA-np4F phoA-np4R	AAGGAGATATACATATGAAGGAAGTCAACAATAT AGGCATTTCTGGAAGCTTTACATAGATATAATTAGAATAT
pET-ncSecP5-mphoA	phoA-np5F phoA-np5R	AAGGAGATATACATATGTCTCAGTTTTTGAGAAA AGGCATTTCTGGAAGCTTTTCCCGACACACACAGA
pET-ncSecP6-mphoA	phoA-np6F phoA-np6R	AAGGAGATATACATATGTCGATTTAAATTGGTTTG AGGCATTTCTGGAAGCTTTCTATCGTTATTATTCTGC
pET-ncSecP7-mphoA	phoA-np7F phoA-np7R	AAGGAGATATACATATGTACTATAACAGCAACACTG AGGCATTTCTGGAAGCTTATCGCTCAAAAAGACAAGA
pET-ncSecP8-mphoA	phoA-np8F phoA-np8R	AAGGAGATATACATATGAGGTCAGTGAACAATA AGGCATTTCTGGAAGCTTCACCTTACAATCTTGATC
pET-ncSecP9-mphoA	phoA-np9F phoA-np9R	AAGGAGATATACATATGAGCGGGTGTCCGATAA AGGCATTTCTGGAAGCTTTAGGTCAGTTTGATCTC
pET-ncSecP10-mphoA	phoA-np10F phoA-np10R	AAGGAGATATACATATGGATATCTATGATGGCTC AGGCATTTCTGGAAGCTTATCTCCATAACTTGGGA
pET-ncSecP11-mphoA	phoA-np11F phoA-np11R	AAGGAGATATACATATGAATATTTAGAAAATTAACAAAT AGGCATTTCTGGAAGCTTCTTGAGATAAAAAGAACGT
pET-ncSecP12-mphoA	phoA-np12F phoA-np12R	AAGGAGATATACATATGTGCAATGTTATTGTTCG AGGCATTTCTGGAAGCTTAAGAGTAAGAGAGTCGT
pET-ncSecP13-mphoA	phoA-np13F phoA-np13R	AAGGAGATATACATGTGATAAAGTCGACAAAGGA AGGCATTTCTGGAAGCTTTCTTTTCTAATTTTGATAGT
pET-ncSecP14-mphoA	phoA-np14F phoA-np14R	AAGGAGATATACATATGAAATCCAGTGATCAGAGCA AGGCATTTCTGGAAGCTTTTGCTGGATTCTTGGA
pET-ncSecP15-mphoA	phoA-np15F phoA-np15R	AAGGAGATATACATTTGCCGTTAAGTGTTATAGC AGGCATTTCTGGAAGCTTTCTTCCATTTTCGGTGG
pET-ncSecP16-mphoA	phoA-np16F phoA-np16R	AAGGAGATATACATATGTTGCGCGATGGATTGCA AGGCATTTCTGGAAGCTTTCTTTTCTAATTTTCCCT
pET-ncSecP17-mphoA	phoA-np17F phoA-np17R	AAGGAGATATACATATGAAAAATTTTTATTGCAATTT AGGCATTTCTGGAAGCTTATAATCAGGAGACCAAGC
pET-ncSecP18-mphoA	phoA-np18F phoA-np18R	AAGGAGATATACATATGACCATTTCTTCTCATC AGGCATTTCTGGAAGCTTTGTGTTGTGTGCCAT
pET-ncSecP19-mphoA	phoA-np19F phoA-np19R	AAGGAGATATACATATGAAAAAGTATTTCACAATTTAAC AGGCATTTCTGGAAGCTTGAGCGGTGTCGGTTGT
pET-ncSecP20-mphoA	phoA-np20F phoA-np20R	AAGGAGATATACATATGTTTTATTGTTGATAT AGGCATTTCTGGAAGCTTTAATCTTTTATTATTATTATC
pET-ncSecP21-mphoA	phoA-np21F phoA-np21R	AAGGAGATATACATATGAGTAAAGTAGAGAAAAAA AGGCATTTCTGGAAGCTTAATCAATCTTTTATTGGT
pET-ncSecP22-mphoA	phoA-np22F phoA-np22R	AAGGAGATATACATATGGAGATTAATCAGGCGTG AGGCATTTCTGGAAGCTTTACGTATCCTTCCGAAGTA
pET-ncSecP23-mphoA	phoA-np23F phoA-np23R	AAGGAGATATACATATGTTAATCCAGAATAACTTGACCT AGGCATTTCTGGAAGCTTTTCTATGATGTTTTGGGT
pET-ncSecP24-mphoA	phoA-np24F phoA-np24R	AAGGAGATATACATATGGATATCAAGGGATTAATAG AGGCATTTCTGGAAGCTTTTTCTTAAAAAATTTGTGAT
pET-ncSecP25-mphoA	phoA-np25F phoA-np25R	AAGGAGATATACATATGCAAGTCCTTCTATTAG AGGCATTTCTGGAAGCTTTTACTTATCTCAGGTAAA
pET-ncSecP26-mphoA	phoA-np26F phoA-np26R	AAGGAGATATACATGTGACGCGCTATATGAATA AGGCATTTCTGGAAGCTTTTCTACCATACCAATAC
pET-ncSecP27-mphoA	phoA-np27F phoA-np27R	AAGGAGATATACATTTGAGTACGACCGCGCATAC AGGCATTTCTGGAAGCTTATTTTTTATCCTTCTGTGTG
pET-ncSecP28-mphoA	phoA-np28F phoA-np28R	AAGGAGATATACATATGGAGAGTGTTTTAGGAGTT AGGCATTTCTGGAAGCTTTCTACCCCTCCAACT
pET-ncSecP29-mphoA	phoA-np29F phoA-np29R	AAGGAGATATACATGTGTCTCTAGTAAGATATGG AGGCATTTCTGGAAGCTTATGATCTGTATAAAGAATGG
pET-ncSecP30-mphoA	phoA-np30F phoA-np30R	AAGGAGATATACATATGGTTTCTCTCTCAGTT AGGCATTTCTGGAAGCTTCGAAGGCAAACTATCAG
pET-ncSecP31-mphoA	phoA-np31F	AAGGAGATATACATATGGCTGAATTAGAAACAGCTC

(Continued on next page)

TABLE 2 (Continued)

Construct	Primer	Primer sequence (5'–3')
pET-ncSecP32-mphoA	phoA-np31R phoA-np32F phoA-np32R	AGGCATTCTGGAAGCTTAGATCGTGCAGTTTTCC AAGGAGATATACATATGGAGACTTTTCATGAGTGAAA AGGCATTCTGGAAGCTTATTCTTTGTTGCGTCTTG
PCD assay		
pPVX-ncSecP1	pvx-np1F pvx-np1R	GAGCACCAGCTAGCATCGATATGGCATATTGGCTTGTA CAAGCTTATCGGCGGTGACCTACAAAGGAGGATTAGATAG
pPVX-ncSecP2	pvx-np2F pvx-np2R	GAGCACCAGCTAGCATCGATATGAAAAACGAGAAAGAAT CAAGCTTATCGGCGGTGACCTAATTCGATTGGAATAGG
pPVX-ncSecP3	pvx-np3F pvx-np3R	GAGCACCAGCTAGCATCGATATGGTCTGGACTGTTGAAAG CAAGCTTATCGGCGGTGACCTAATTCGAATTTGCCTGAAC
pPVX-ncSecP5	pvx-np5F pvx-np5R	GAGCACCAGCTAGCATCGATATGTCTCAGTTTTTGAGAAA CAAGCTTATCGGCGGTGACCTATTTCCGACACACACAGA
pPVX-ncSecP6	pvx-np6F pvx-np6R	GAGCACCAGCTAGCATCGATATGTCGTATTTAAATTGGTTTG CAAGCTTATCGGCGGTGACCTATCTATCGTTATTATTCTGC
pPVX-ncSecP8	pvx-np8F pvx-np8R	GAGCACCAGCTAGCATCGATATGAGGTCAGTGCAACAATA CAAGCTTATCGGCGGTGACCTACACCTTACAATCTTGATC
pPVX-ncSecP9	pvx-np9F pvx-np9R	GAGCACCAGCTAGCATCGATATGAGCGGGTGTCCGATAA CAAGCTTATCGGCGGTGACCTATAGGTGAGTTTGTATCTC
pPVX-ncSecP10	pvx-np10F pvx-np10R	GAGCACCAGCTAGCATCGATATGGATATCTATGATGGCTC CAAGCTTATCGGCGGTGACCTAATCTCCATAACTTGGGA
pPVX-ncSecP12	pvx-np12F pvx-np12R	GAGCACCAGCTAGCATCGATATGTGCAATGTTATTGTTTCG CAAGCTTATCGGCGGTGACCTCAAAGAGTAAGAGAGTCGT
pPVX-ncSecP13	pvx-np13F pvx-np13R	GAGCACCAGCTAGCATCGATATGTGATAAAGTCGACAAAGGA CAAGCTTATCGGCGGTGACCTAACATTCACCTAAATTTGTAGAT
pPVX-ncSecP14	pvx-np14F pvx-np14R	GAGCACCAGCTAGCATCGATATGAAATCCAGTGATCAGAGCA CAAGCTTATCGGCGGTGACCTATTGCTTGGATTCTTGGA
pPVX-ncSecP15	pvx-np15F pvx-np15R	GAGCACCAGCTAGCATCGATTTGCCGTTAAGTGGTTATAGC CAAGCTTATCGGCGGTGACCTATTCTTCCATTTTCGGTGG
pPVX-ncSecP16	pvx-np16F pvx-np16R	GAGCACCAGCTAGCATCGATATGTTGCGCGATGGATTGCA CAAGCTTATCGGCGGTGACCTATTCTTTTCTAATTTTCCCT
pPVX-ncSecP17	pvx-np17F pvx-np17R	GAGCACCAGCTAGCATCGATATGAAAAATTTTATTGCAATTT CAAGCTTATCGGCGGTGACCTAATAATCAGGAGACCAAGC
pPVX-ncSecP19	pvx-np19F pvx-np19R	GAGCACCAGCTAGCATCGATATGAAAAAGTATTTACAATTTAAC CAAGCTTATCGGCGGTGACCTAGTAGCGGTGTCCGTTGT
pPVX-ncSecP20	pvx-np20F pvx-np20R	GAGCACCAGCTAGCATCGATATGTTTTATTTTTTGTGGATAT CAAGCTTATCGGCGGTGACCTATAACTTTTTATTATTATTATC
pPVX-ncSecP21	pvx-np21F pvx-np21R	GAGCACCAGCTAGCATCGATATGAGTAAAGTAGAGAAAAA CAAGCTTATCGGCGGTGACCTAAATCAATCCTTTTATTGGT
pPVX-ncSecP22	pvx-np22F pvx-np22R	GAGCACCAGCTAGCATCGATATGGAGATTAATCAGGCGTG CAAGCTTATCGGCGGTGACCTATACGTATCCTCCGAAGTA
pPVX-ncSecP23	pvx-np23F pvx-np23R	GAGCACCAGCTAGCATCGATATGTTAATCCAGAATAACTTGACCT CAAGCTTATCGGCGGTGACCTATCATGATGCTTTTGGGT
pPVX-ncSecP24	pvx-np24F pvx-np24R	GAGCACCAGCTAGCATCGATATGGATATCAAGGGATTAATAG CAAGCTTATCGGCGGTGACCTATTTTCTAAAAAATTTGTGAT
pPVX-ncSecP25	pvx-np25F pvx-np25R	GAGCACCAGCTAGCATCGATATGCAAGTCCTTCTATTAG CAAGCTTATCGGCGGTGACCTATTACTATCTCAGGTAAA
pPVX-ncSecP26	pvx-np26F pvx-np26R	GAGCACCAGCTAGCATCGATGTGCAGCGGTATATGAATA CAAGCTTATCGGCGGTGACCTATGTTCTACCATAACCAATAC
pPVX-ncSecP27	pvx-np27F pvx-np27R	GAGCACCAGCTAGCATCGATTTGAGTACGACCGCGCATAC CAAGCTTATCGGCGGTGACCTAATTTTTTTATCCTTCTGTGTG
pPVX-ncSecP28	pvx-np28F pvx-np28R	GAGCACCAGCTAGCATCGATATGGAGAGTGTTTTAGGAGTT CAAGCTTATCGGCGGTGACCTCATCTACCCCTCCAACT
pPVX-ncSecP30	pvx-np30F pvx-np30R	GAGCACCAGCTAGCATCGATATGGTTTCTCCTCCTCAGTT CAAGCTTATCGGCGGTGACCTACGAAGGCAAACTATCAG
pPVX-ncSecP31	pvx-np31F pvx-np31R	GAGCACCAGCTAGCATCGATATGGCTGAATTAGAAACAGCTC CAAGCTTATCGGCGGTGACCTAAGATCGTGCAGTTTTCC
pPVX-ncSecP32	pvx-np32F pvx-np32R	GAGCACCAGCTAGCATCGATATGGAGACTTTCATGAGTGAAA CAAGCTTATCGGCGGTGACCTAATTTCTTGTGCGTCTTG
pPVX-PR1	pvx-pr1F pvx-pr1R	GAGCACCAGCTAGCATCGATATGGGATTTGTTCTCTTTCC CAAGCTTATCGGCGGTGACCTAGTATGGACTTTGGCCAAAT
pPVX-PR2	pvx-pr2F pvx-pr2R	GAGCACCAGCTAGCATCGATATGGCTTTATGCATTAATAATG CAAGCTTATCGGCGGTGACCTAATTAATAATTGATATTTTGC

(Continued on next page)

TABLE 2 (Continued)

Construct	Primer	Primer sequence (5'–3')
pVX-PR5	pvx-pr5F pvx-pr5R	GAGCACCAGCTAGCATCGATATGAACCTCCTCAAAGCTTCC CAAGCTTATCGGCGGTGCGACTCAAGGGCAGAAGACAACCT
Sublocalization assay		
pCAM-ncSecP1-GFP	gfp-np1F gfp-np1R	GACGAGCTCGGGTACCATTGGTGGCATATTGGCTTGTAAG TGGTGTGCGACTCTAGACAAAGGAGGATTAGATAG
pCAM-ncSecP3-GFP	gfp-np3F gfp-np3R	GACGAGCTCGGGTACCATTGGTCTGGACTGTTGAAAG TGGTGTGCGACTCTAGATTCTGAATTTGCCTGAAC
pCAM-ncSecP12-GFP	gfp-np12F gfp-np12R	GACGAGCTCGGGTACCATTGTGCAATGTTATTGTTTCG TGGTGTGCGACTCTAGAAAGAGTAAGAGAGTCGT
pCAM-ncSecP15-GFP	gfp-np15F gfp-np15R	GACGAGCTCGGGTACCATTGTTGCCGTTAAGTGGTTATAGC TGGTGTGCGACTCTAGATTCTTCCATTTTCGGTGG
pCAM-ncSecP22-GFP	gfp-np22F gfp-np22R	GACGAGCTCGGGTACCATTGGAGATTAATCAGGCGTG TGGTGTGCGACTCTAGATACGTATCCTTCCGAAGTA
pCAM-ncSecP23-GFP	gfp-np23F gfp-np23R	GACGAGCTCGGGTACCATTGTTAATCCAGAATAACTTGACCT TGGTGTGCGACTCTAGATTCTATGATGCTTTTGGGT
pCAM-ncSecP24-GFP	gfp-np24F gfp-np24R	GACGAGCTCGGGTACCATTGGATATCAAGGGATTAATAG TGGTGTGCGACTCTAGACTGTCTAAAGATATCTTTCTG
pCAM-ncSecP26-GFP	gfp-np26F gfp-np26R	GACGAGCTCGGGTACCATTGGTGACGCGGTATATGAATA TGGTGTGCGACTCTAGATGTTCTACCATAACCAATAC
pCAM-ncSecP27-GFP	gfp-np27F gfp-np27R	GACGAGCTCGGGTACCATTGTTAGTAGACACCGCGCATAC TGGTGTGCGACTCTAGAATTTTTTTATCCTTCTGTGTG
pCAM-ncSecP28-GFP	gfp-np28F gfp-np28R	GACGAGCTCGGGTACCATTGGAGAGTGTTTTAGGAGTT TGGTGTGCGACTCTAGATCTACCCCTCCAACT
PCR detection of CLAs		
16s rRNA	CLas16sF CLas16sR	GCGCGTATGCAATACGAGCGGCA GCCTCGGACTTCGCAACCCAT

PhoA activities of the transformants were visualized using indicator LB agar (supplemented with 90 μ g/ml BCIP [5-bromo-4-chloro-3-indolylphosphate], 100 mM IPTG [isopropyl- β -D-thiogalactopyranoside], and 75 mM Na_2HPO_4) at 37°C after up to 36 h of incubation. The negative control containing empty pET-mphoA and the positive control with pET-phoA bearing the full-length *phoA* were also used for validation.

PCD suppression assay and DAB staining. The genes encoded by the selected ncSecPs in addition to *PR-1*, *PR-2*, and *PR-5* from *N. benthamiana* were amplified with the primers shown in Table 2 and then inserted into Clal/Sall-treated pGR107, which is a binary plant expression vector based on *Potato virus X* (PVX) (93). The resulting recombinant PVX vectors, including pPVX-ncSecPn (wherein “n” represents an integer from 1 to 27), pPVX-PR-1, pPVX-PR-2, and pPVX-PR-5, were individually introduced into *Agrobacterium tumefaciens* GV3101 and subjected to PCD suppression assays via agroinfiltration, as previously described (94). Briefly, agrobacterial cells carrying pPVX-ncSecPn, pPVX-PR-1, pPVX-PR-2, or pPVX-PR-5 were initially inoculated into expanded leaves of 6-week-old *N. benthamiana* plants, with the agrobacterial cells harboring pPVX-GFP as a negative control. At 24 h postinoculation (hpi), the infiltration sites were further challenged with agrobacterial cells carrying pPVX-Bax or pPVX-INF1. At 2 days after *Bax* or *INF1* infiltration, one-third of the infiltrated leaves were removed for histochemical staining with DAB, as previously described (95). Development of cell death was recorded at 5 days after *Bax* or *INF1* infiltration. Each assay comprised at least six plants with two leaves being inoculated and was repeated two times. The PCD suppression ability of each ncSecP was then evaluated with the cell death suppression number/infiltration number (CDS/I) index, which was recorded as consistent suppression (CDS/I > 90%), partial suppression (90% $\leq$ CDS/I $\leq$ 50%), or no suppression (CDS/I < 50%).

Confocal imaging. ncSecP coding sequences were amplified with the primers listed in Table 2 and cloned into pCAMBIA1300-35S-GFP that was digested with KpnI and XhoI. The resulting construct contained the fusion gene *ncSecP-gfp* that was further introduced into *A. tumefaciens* EHA105, followed by agroinfiltration of leaves of the 4-week-old *N. benthamiana* plants. Transiently expressed GFP fusions were observed using an LSM700 confocal microscope (Zeiss, Germany) at 60 hpi. GFP was excited at 488 nm, and fluorescence emission was detected between 500 and 530 nm. Red fluorescence protein was excited at 543 nm and captured between 590 and 630 nm.

Sample preparation and total RNA extraction. CLas-infected Valencia sweet orange (*C. sinensis* Osbeck) seedlings and CLas-bearing psyllids were prepared as previously described (41). Total RNA was extracted from the midribs of citrus leaves or psyllids (12 were pooled as a single sample) using the TRIzol reagent (Invitrogen) according to the manufacturer's instructions.

The fully expanded *N. benthamiana* leaves at the five- to six-leaf stage were first inoculated with *A. tumefaciens* GV3101 cells expressing ncSecPm (wherein “m” represents 1, 3, 12, 15, 22, 23, 24, 26, 27, or 28) or GFP and were further challenged with *Bax* or *INF1* at 24 hpi, as described above under “PCD suppression assay and DAB staining.” At 24 h after *Bax* or *INF1* infiltration, the double-infiltrated tissues were

TABLE 3 Primers used for RT-qPCR analysis^a

Gene	Primer	Primer sequence (5'–3')
<i>LasgyrA</i>	gyrase296-F	GTATGGCACAGGACTGGTCT
	gyrase486-R	GTTAGGGCGGAAATCAACAGT
<i>ncSecP1</i>	np1F	TGCAACAAGATAAGGGGCGT
	np2R	TCCACACATCCCCAACACGA
<i>ncSecP3</i>	np3F	CAGCTCCCAGTTGTTAGGTCT
	np3R	GGGGAATCATTGCACACATCG
<i>ncSecP12</i>	np12F	CCCAAAGACACCAGCGATAC
	np12R	TTTAGAGGAGAGGATTGCTTGG
<i>ncSecP15</i>	np15F	CGAAATCAGCCCGTCATAGA
	np15R	TTTCTGATTGCTGCTTGGG
<i>ncSecP22</i>	np22F	TAGGGATTGGCAAAGAATAGC
	np22R	GCCAAAGAAGCGTTATCTCTATT
<i>ncSecP23</i>	np23F	GTTAATCCAGAATAACTTGACC
	np23R	TGATGCTTTTGGGTTCTTTCT
<i>ncSecP24</i>	np24F	ATAATGAGTGGGTGTTCCGA
	np24R	TATCTTCTGCCACCACCTT
<i>ncSecP26</i>	np26F	TGATGGCTGCTCTATTGCTG
	np26R	TAGGCGTCACTGGTCTCGTA
<i>ncSecP27</i>	np27F	TTTGCGACACATCTTCTCTCC
	np27R	CTGTGTGATACTCGGATGCG
<i>ncSecP28</i>	np28F	TTCTACTCCCAATAATCTCCC
	np28R	CTCGTTTCTCCAGATTCA
<i>NbPR-1</i>	NbPR1F	AACCTTTGACCTGGGACGAC
	NbPR1R	AGCACATCCAACACGAACTG
<i>NbPR-2</i>	NbPR2F	CATTAGCAGCAGCAGGGTTG
	NbPR2R	GAGTGAAGGTTATGTCGTGC
<i>NbPR-5</i>	NbPR5F	GAACAGTCCAGGCTCGCATA
	NbPR5R	GCATCTAAGGTTACGGCACC
<i>Nbactin</i>	NbActin-F	GCAGGAATCCACGAGACTACA
	NbActin-R	AACCTCCAATCCAGACACTGT
<i>CsPR-1</i>	CsPR1F	AAATGTGGGTGAATGAGAAAGC
	CsPR1R	ATTATTGTTGCACGTCACCTTG
<i>CsPR-2</i>	CsPR2F	TTCCAATGCCATCGAAACTG
	CsPR2R	GTAATCTTGTTTAAATGAGCCTCTTG
<i>CsPR-5</i>	CsPR5F	CACCATTGCCAATAACCCCTAATG
	CsPR5R	GGGACAGTTACCGTTAAGATCAG
<i>Csactin</i>	CsActin-F	CATCCCTCAGCACCTTCC
	CsActin-R	CCAACCTTAGCACTTCTCC

^aThe nucleotide sequences of *NbPR1* (Niben101Scf13926g01014.1), *NbPR2* (Niben101Scf01001g00005.1), and *NbPR5* (Niben101Scf01400g00014.1) were retrieved from *N. benthamiana* draft genome sequence V1.0.1 (https://solgenomics.net/organism/Nicotiana_benthamiana/genome) and used for the design of the primers listed here. The primers for *CsPR1*, *CsPR2*, and *CsPR5* were designed according to previously published reports (91, 92).

harvested. Meanwhile, the *N. benthamiana* leaves were infiltrated with *A. tumefaciens* GV3101 cells expressing Bax, INF1, or GFP and were harvested at 24 hpi. The collected leaf tissues were subjected to total RNA extraction by using an RNeasy minikit (Qiagen).

Sour pomelo and Jincheng orange plants were prepared and infected with CLas by grafting, as previously described (96). Two-month-old asymptomatic leaves were collected from CLas-infected citrus plants and then subjected to PCR analysis to ensure the presence of CLas, as described by Zou et al. (97). Leaves of healthy plants exhibiting positions and developmental stages similar to those of the asymptomatic leaves were used as controls. Total RNA was extracted from the leaves by using an RNeasy minikit.

RT-qPCR analysis. Total RNA (1 µg) was reverse transcribed into first-strand cDNA with random primers by using a PrimeScript RT reagent kit with gDNA Eraser (TaKaRa, Japan). SYBR Green-based PCR assays were then performed using TBGreenPremix EX TaqII (TaKaRa) and gene-specific primers (Table 3) on an ABI StepOne plus real-time PCR instrument (Applied Biosystems, Foster City, CA), as previously described (39). The CLas DNA gyrase subunit A (*LasgyrA*) gene (GenBank no. CP001677.5) was used as an internal reference to evaluate the expression of *ncSecP*-encoding genes in citrus plants and psyllids. The *N. benthamiana* actin (GenBank no. JQ256516.1) and citrus actin (GenBank no. GU911361.1) genes were used as references for the expression of *N. benthamiana* and citrus plant qPCR assays, respectively. Each reaction was conducted with three biological replicates (each consisting of three technical replicates), along with no-template controls and no-reverse transcription controls. The relative gene expression values were calculated using the C_T

method ($2^{-\Delta\Delta CT}$) (98) and subsequently transformed to fold change values. Data were statistically analyzed using Student's *t* tests (SPSS 10.0; SPSS, Chicago, IL).

Data availability. All relevant data are included in the content of the manuscript.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, XLS file, 0.04 MB.

SUPPLEMENTAL FILE 2, PDF file, 0.4 MB.

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