A "Candidatus Liberibacter asiaticus"-secreted polypeptide suppresses plant immune responses in *Nicotiana benthamiana* and *Citrus sinensis*

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Citrus Huanglongbing (HLB), known as the most economically devastating disease in citrus industry, is mainly caused by phloem-restricted Gram-negative bacterium "Candidatus Liberibacter asiaticus" (CLas). To date, CLas is still unculturable in vitro, which has been dramatically delaying the research on its pathogenesis, and only few Sec-dependent effectors (SDEs) have been identified to elucidate the pathogenesis of CLas. Here, we confirmed that a CLas-secreted Sec-dependent polypeptide, namely SECP8 (CLIBASIA_05330), localized in nucleus, cytoplasm and cytoplasmic membrane, and showed remarkably higher transcript abundance in citrus than in psyllids. Potato virus X (PVX)-mediated transient expression assays indicated that mSECP8 (the mature form of SECP8) suppressed pro-apoptotic mouse protein BAX and *Phytophthora infestans* elicitin INF1-triggered hypersensitive response (HR) associated phenotypes, including cell death, \( \text{H}_2\text{O}_2 \) accumulation and callose deposition. Intriguingly, mSECP8 also inhibited SDE1 (CLIBASIA_05315)-induced water-soaked and dwarfing symptoms in *Nicotiana benthamiana*. In addition, mSECP8 can promote the susceptibility of transgenic Wanjincheng orange (*Citrus sinensis*) to CLas invasion and further HLB symptom development, and it contributes to the proliferation of *Xanthomonas citri* subsp. *citri* (Xcc). Moreover, the expression of ten immunity-related genes were significantly down-regulated in mSECP8 transgenic citrus than those in wide-type (WT) plants. Overall, we propose that mSECP8 may serve as a novel broad-spectrum suppressor of plant immunity, and provide the first evidence counteractive effect among CLas effectors. This study will enrich and provide new evidences for elucidating the pathogenic mechanisms of CLas in citrus host.

**KEYWORDS**

huanglongbing (HLB), CLas, PCD suppressor, effector, HR, plant immunity, SDE1 (CLIBASIA_05315)
Introduction

Huanglongbing (HLB, also referred to as citrus greening), is the most economically destructive disease of citrus (Bové, 2006; Wang, 2019). The causative agents of HLB consist of three species, namely “Candidatus Liberibacter asiaticus” (CLas), “Ca. L. africanus” (CLaf), and “Ca. L. americanus” (CLam), which are the Gram-negative bacteria, belonging to the phloem-limited, alpha-proteobacteria (Jagoueix et al., 1994; Teixeira et al., 2005; Bové, 2006). Among them, CLas transmitted by Asian citrus psyllid (ACP, Diaphorina citri), dominantly threatens citrus production because of its wide distribution and strong pathogenicity (Coletta-Filho et al., 2004; Haapalainen, 2014).

In general, host plants respond to pathogen infections through two-branched innate immune systems, namely, pathogen-associated molecular patterns (PAMP)-triggered immunity (PTI) and effector-triggered immunity (ETI), generating a “zig-zag” model in plant immune system (Jones and Dangl, 2006; Boller and Felix, 2009; Yuan et al., 2021). Many extracellular plant bacterial pathogens use a type III secretion system (T3SS) to deliver virulence-associated effectors to suppress the plant innate immunity (Yamane et al., 2004; Jones and Dangl, 2006; Wang et al., 2011; Toruño et al., 2016). However, the insect-transmitted and intracellular Gram-negative bacteria, such as CLas, lack T3SS, but can employ a complete Sec-dependent secretion pathway to secret effectors (Duan et al., 2009; Green and Mecsas, 2016; Wang et al., 2017).

When invading into plant host cells, CLas induces a series of immune responses, for instance, induction of pathogenesis-related (PR) genes, callose deposition, reactive oxygen species (ROS) burst and enhanced level of salicylic acid (SA) (Wang, 2019; Pang et al., 2020; Ibanez et al., 2022).

Previous studies have confirmed that many CLas-associated Sec-dependent effectors (SDEs) as virulence factors can suppress or trigger plant immune responses (Jain et al., 2015, 2018; Pitino et al., 2016, 2018; Clark et al., 2018; Liu et al., 2019; Zhang et al., 2019, 2020; Pang et al., 2020; Du et al., 2021; Du J. et al., 2022; Du M. et al., 2022). For instance, non-classical secretory proteins (ncSecPs) SC2_gp095 and Lasbcp (CLIBASIA_RS00445), down-regulation the expression of RbohB gene associated with H2O2-mediated defense signaling in plants (Jain et al., 2015, 2018). m3875 (CLIBASIA_03875) and m4405 (CLIBASIA_04405) function as two hypersensitive response (HR)-based cell death suppressors in Nicotiana benthamiana (Zhang et al., 2019, 2020). SDE15 (CLIBASIA_04025) inhibits plant immunity extensively through interacting with citrus susceptibility gene CaACD2 (ACCELERATED CELL DEATH 2, ACD2). Intriguingly, ten ncSecPs were identified as cell death suppressors that induce the expression of PR-1, PR-2, and PR-5 in N. benthamiana (Du et al., 2021). Systemic acquired resistance (SAR)-related genes were modulated, and CLas colonization was promoted in transgenic citrus by CaLasSDE115 (CLIBASIA_05115) (Du M. et al., 2022).

Only a few CLas secretory proteins trigger HR in plants. SDE1 (CLIBASIA_05315) induces strong callose deposition, cell death and starch accumulation in N. benthamiana, and inhibits the activities of defense genes papain-like cysteine proteases (PLCPs) in citrus (Pitino et al., 2016, 2018; Clark et al., 2018). Prophage-encoded ncSecP AGH17470 triggers PR-associated and salicylic acid (SA)-signaling pathway genes expression and then enhances the resistance of citrus to Xanthomonas citri subsp. citri (Du J. et al., 2022).

Candidatus Liberibacter asiaticus encodes a large number of Sec-dependent proteins with unknown functions, and currently only a few has been characterized. In this study, a Sec-dependent protein SECP8 was identified through bioinformatics analyses and E. coli alkaline phosphatase (PhoA) assay. Its function was characterized by transient expression in N. benthamiana and overexpression in Wanjincheng orange (Citrus sinensis). The results reveal that mSECP8 is a virulence factor by suppressing plant immunity to promote CLas invasion.

Materials and methods

Plant materials, microbial strains, and growth conditions

Wild-type (WT) N. benthamiana plants were grown in a growth chamber maintained at 25 ± 2°C and 16 h light/8 h dark photoperiod. Plants at the five-leaf stage were used for infiltration. The transformant Escherichia coli strains Mach-T1 and Rosetta-gami2 (DE3) were grown on Luria-Bertani (LB) medium at 37°C, and Agrobacterium tumefaciens strains GV3101, GV3101 (pJIC SA_Rep) and EHA105 were cultured on LB medium with 50 µg/mL kanamycin and 20 µg/mL rifampicin at 28°C.

Total RNA extraction, cDNA synthesis, and quantitative PCR analysis

The collected leaf samples were stored in −80°C refrigerator. Total RNA was extracted with RNAiso Plus (Takara) reagent and its concentration was quantified. Then 1 µg total RNA was reverse transcribed with All-in-One 5 × RT MasterMix Reagent Kit (ABM) in a 20 µL reaction. The cDNA was diluted five times and then used for quantitative PCR (qPCR) assay in a 10 µL reaction, consisting of 5 µL of Blastaq™ 2 × qPCR MasterMix (ABM), 3.5 µL of DNase/RNase free water, 0.25 µL of each primer (10 µM), and 1 µL of diluted cDNA template. The PCR cycling consisted of an initial activation step at 95°C for 3 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The CLas gyrA gene (CLIBASIA_00325) and citrus actin gene (XM_006464503.3) were chosen as internal controls. The relative expression assay
of genes was performed with three biological and technical replicates, and calculated using the 2−ΔΔCt method (Livak and Schmittgen, 2001). Primers used were detailed (Supplementary Table 1).

DNA extraction and Candidatus Liberibacter asiaticus detection analysis

For CLas detection, total DNA was extracted from the newly emerged CLas-inoculated leaves using the Biospin Omni Plant Genomic DNA Extraction Kit (BioFlux). PCR was performed with 2 × Taq Master Mix (Novoprotein) in a 12 μL reaction. The qPCR ran in a 10 × reaction, consisting of 5 μL of Blastaq™ 2 × qPCR MasterMix (ABM), 3.5 μL of DNase/RNase free water, 0.25 μL of each primer (10 μM), and 1 μL of DNA (200 ng/μL). The cycling comprised an initial activation step at 95°C for 3 min, followed by 40 cycles of 95°C for 15 s, 60°C for 15 s and 72°C for 15 s.

Bioinformatics analysis

The SECP8 sequence was extracted from the genome of CLas strain Psy62 (GenBank No. CP001677.5) (Duan et al., 2009). Sequences of other CLas strains including AHCA1 (GenBank No. CP029348.1), YNJ57C (GenBank No. QXDO00000000.1), Gxpsy (GenBank No. CP004005.1), A4 (GenBank No. CP010804.1), YCPsy (GenBank No. LIIM00000000.1), JXGC (GenBank No. CP019958.1), and Ishi-1 (GenBank No. AP014595.1) were also downloaded from NCBI1. The signal peptide (SP) and the transmembrane domain were predicted with SignalP 3.0 using neural networks (NN) method (Bendtsen et al., 2004) and TMHMM v2.0 (Krogh et al., 2001), respectively. DNAMAN software was used for sequence alignment.

3,3′-diaminobenzidine trypan-blue and aniline-blue staining assays

For symptoms visualization, the infiltrated leaves were immersed in 95% ethanol (v/v) for 48 h at 37°C until the leaves became clear.

For H2O2 accumulation assay, the infiltrated leaves were sampled and soaked in the 3,3′-Diaminobenzidine (DAB) staining buffer (pH 3.0; 1 mg/mL DAB, 0.05% Tween20 and 10 mM Na2PO4) for 12 h in the dark (Thordal-Christensen et al., 1997; Vanacker et al., 2000). Then the leaves were transferred into boiled bleaching solution (20% acetic acid, 20% glycerin, and 60% ethanol) for discoloration.

For programmed cell death (PCD) observation, the injected leaves were soaked in the trypan-blue staining solution (0.067% w/v trypan-blue, 11.11% w/v phenol, 1 vol of glycerol, 1 vol of lactic acid, 1 vol of double distilled water, 6 vol of 95% ethanol) and boiled for 2 min. Then the samples were transferred into chloral hydrate (1.25 g/mL) and shaken at 50 rpm until leaves were fully de-stained (Heese et al., 2007).

For callose deposition assay, the plant leaves were incubated in solution (acetic/glycerin/ethanol, 1v/1v/3v) overnight until the green color faded away, then washed with 150 mM K2HPO4 for 30 min. Finally, samples were stained with aniline-blue solution (150 mM K2HPO4, 0.05% w/v aniline-blue), and the callose deposition was observed via fluorescence microscope (Sebastian and Adam, 2015).

Agrobacterium-mediated transient expression assay in Nicotiana benthamiana

The coding sequence of mSECP8, SECP8, and SDE1 were amplified with corresponding primers (Supplementary Table 1), and cloned into Clal/Sall linearized pGR107 vector (a Potato virus X-based expression vector, PVX) to generate experimental recombinant plasmids. These constructs were then transferred into A. tumefaciens GV3101 (pJIC SA_Rep). The PCD suppression assay was performed as described previously (Wang et al., 2011). Briefly, inoculation buffer (10 mM MES, pH 5.6, 10 mM MgCl2, and 100 μM acetosyringone [AS]) or A. tumefaciens cells harboring PVX-SECP8, PVX-mSECP8 and PVX-eGFP (negative control) with OD600 adjusting to 1.0 were firstly infiltrated into the newly expanded leaves of N. benthamiana plants. After 24 h, A. tumefaciens-mediated PVX-BAX, PVX-INF1 or PVX-SDE1 was infiltrated into the same area of leaves. At 48 h post inoculation (hpi), partial infiltrated leaves were used for H2O2 accumulation assay, while the remaining leaves were used for observation of the cell death phenotypes up to 7 days post inoculation (dpi). The suppression effects of SECP8 on SDE1 transient expression plants were evaluated at 15 dpi.

Alkaline phosphatase assay

The signal peptide sequence of SECP8 (SECP8SP) was amplified with the specific primers (Supplementary Table 1) and cloned into pET-mphoA vector. The recombinant plasmids pET-mphoA (negative control), pET-phoA (positive control) and pET-SECP8SP-mphoA were transformed into E. coli Rosetta-gami2 (DE3), then the E. coli cells were grown at 37°C for 12 h on indicator LB solid medium (with 90 μg/mL 5-bromo-4-chloro-3-indolyl phosphate [BCIP], 1 https://www.ncbi.nlm.nih.gov/genome/browse/#!/prokaryotes/1750/
Subcellular localization of mSECP8 in Nicotiana benthamiana

The coding sequence of mSECP8 was amplified with specific primers (Supplementary Table 1) and ligated into KpnI/XbaI double-digested pCAMBIA1300-35S-eGFP vector. The infiltration of the experimental recombinants into N. benthamiana leaves was mediated by A. tumefaciens EHA105, meanwhile, plasma membrane marker pm-rk CD3-1007 (mcherry) (Nelson et al., 2007) and nuclear marker H2B-RFP (Liu et al., 2019) were co-infiltrated. The inoculated leaves were collected after 2 dpi and observed with confocal laser scanning microscopy.

Genetic transformation of Wanjincheng orange (Citrus sinensis)

The coding region of mSECP8 was obtained by PCR with the designated primers (Supplementary Table 1), subsequently inserted into BamH1/EcoRI-digested pCAMBIA-GN-35S-MCS-NOS (pLGN) vector. To obtain the transgenic plants, the epicotyl explants of Wanjincheng orange were used for A. tumefaciens-mediated transformation (Peng et al., 2015; Zou et al., 2017). The successful transgenic lines were identified with both GUS histochemical staining and reverse transcription-PCR (RT-PCR) assays. All transgenic and WT control plants were grafted onto Poncirus trifoliata for following evaluation.

The susceptibility evaluations of transgenic citrus to huanglongbing and canker

Half-year-old transgenic and WT Wanjincheng plants were graft inoculated with CLas positive buds by qPCR verification. The establishment of CLas on transgenic plants was firstly assayed at 40 dpi by both PCR and qPCR (Jagoueix et al., 1996; Li et al., 2006), with the cytochrome oxidase gene of Citrus sinensis as internal control, then detected monthly afterward. All samples were performed with three biological and technical replicates. The primers used were listed in Supplementary Table 1.

To assess the susceptibility of transgenic Wanjincheng plants to Xcc, the in vitro pinprick inoculation was carried out as previously described (Peng et al., 2015, 2017; Zou et al., 2021). Four leaves per transgenic line and WT control were tested, and per leaf contained six infected sites of which each comprised six punctures in 0.5 mm diameter by a needle, 1 μL bacterial Xcc suspension (2.5 × 10^8 CFU/mL) was subsequently dropped onto each puncture site. Symptom Photographs were taken at 12 dpi, and the lesion sizes were assessed by ImageJ software. For each transgenic lines and WT plants, the disease indexes of 36 punctures per leaf were calculated depending on the following rating index: 1, < 0.75 mm^2 (lesion size); 2, 0.75–1.25 mm^2; 3, 1.25–1.75 mm^2; 4, 1.75–2.25 mm^2; 5, 2.25–2.75 mm^2; 6, 2.75–3.25 mm^2; 7, >3.25 mm^2. The disease index (DI, %), indicating the level of susceptibility to Xcc, was calculated using the formula (Peng et al., 2017):

\[ DI = \frac{\sum \text{[number of each index × the corresponding index]}}{\text{[number of lesions × the max index]}} \times 100 \]

At 10 dpi, to count the number of Xcc colonies, the individual disease spots of inoculated leaves were detached using a 5-mm diameter size hole puncher. Three leaf discs were collected together and firstly ground in 200 μL sterile double distilled water (ddH_2O), then added another 800 μL ddH_2O. The suspension was mixed by 10 times gradient dilution (10^{-3} to 10^{-5}) and then plated on LB medium. After incubation at 28°C for 48 h, Xcc colonies were counted, and the number of bacterial cells (CFU) per square centimeter of leaf tissue was calculated. Each specific dilution contained three replicates and the test was repeated three times. The bacterial strain Xcc-29-1 used in this examination was provided by Professor Gongyou Chen (School of Agriculture and Biology, Shanghai Jiao Tong University, Shanghai, China).

Results

SECP8 encodes a conserved sec-delivered polypeptide with significantly higher expression level in citrus than in psyllids

In silico analysis of SECP8 (CLIBASIA_05330) indicated that the polypeptide is comprised of 67 amino acids (aa) with an 18-aa predicted signal peptide (namely SECP8SP) in the N-terminal (Figure 1A and Supplementary Figure 1B), and SECP8 was predicted without transmembrane domain (Supplementary Figure 1C). A BLASTn search from the GenBank database revealed that SECP8 encoded a hypothetical protein with 100% similarity to the sequence from all available CLas strains, among them, the SECP8 homologs from eight representative strains were employed for sequence alignments (Supplementary Figure 1A), while the homology of SECP8
could not be found in CLaf and CLam genomes, suggesting SECP8 is unique in CLas strains.

Through phoA assay, the E. coli (Rosetta-gami2 DE3 strain) carrying the fusion protein SECP8SP-mphoA turned blue after 12 h of incubation on indicator LB medium as the positive control, while the negative control construct remained white (Figure 1B). Taken together, the phoA assays along with bioinformatics analysis indicated SECP8 as a typical Sec-delivered polypeptide.

To determine the subcellular localization of SECP8, the infiltrated leaves were collected and observed by confocal fluorescence microscopy. Similar to the distribution of eGFP control, the green fluorescence signals of mSECP8 was observed in the nucleus, cytoplasm and cytoplasmic membrane as the membrane and nucleus markers (Figure 1C).

The expression pattern of mSECP8 was further analyzed in CLas-infected citrus and psyllids by reverse transcription-quantitative PCR (RT-qPCR). The transcript level of SECP8 was significantly higher (~12-fold) in citrus host than in psyllids vectors (Figure 1D), suggesting that SECP8 may play a more important role in planta.

mSECP8 suppresses BAX- and INF1-triggered hypersensitive cell death in Nicotiana benthamiana

The mouse BAX and the P. infestans INF1 are well-known as cell death elicitors and widely applied to identify the PCD suppressor of pathogens (Kamoun et al., 1998; Lacomme and Cruz, 1999). Therefore, the effect of mSECP8 on PCD suppression was evaluated with the strategy. At 7 dpi, both SECP8 and mSECP8 suppressed BAX- and INF1-induced cell death in N. benthamiana, whereas the phenomenon has not been observed in eGFP and buffer infiltration (Figures 2A,B). The result was confirmed by ethanol decolorization and trypan-blue staining assays (Figures 2C,D).

To further explore the role of mSECP8 on cell death suppression, the accumulation of H2O2 as a marker of plant cell death was assayed in N. benthamiana leaves by DAB staining. We found that mSECP8 significantly inhibited H2O2 burst triggered by both BAX- and INF1 (Figure 2E). In contrast, neither eGFP nor the infiltration buffer interfered with H2O2 accumulation.
Callose deposition as another indicator during plant immunity responses was examined. At 2−3 dpi, mSECP8 dramatically reduced BAX-triggered callose deposition compared to eGFP control, particularly at 3 dpi (Figure 2F). Herein, these results clearly demonstrate that SECP8 is a PCD suppressor.

**mSECP8 inhibits SDE1 induced water-soaked necrosis and plant dwarfing in Nicotiana benthamiana**

Previously, SDE1 (CLIBASIA_05315) was identified as a CLas Sec-dependent effector and induced water-soaked necrosis in *N. benthamiana* at 4 dpi (Pitino et al., 2016, 2018). Then we examined whether the PCD suppressor SECP8 interferes SDE1 function. We observed that SDE1 induced water-soaked necrosis was present at injection areas at 4 dpi (Figure 3C) and the plants even displayed severe necrosis, dwarfing, leaf curling and shrinking phenotypes at 15 dpi (Figures 3A,B). Similar symptoms were also observed in *N. benthamiana* infected with *eGFP + SDE1* and *buffer + SDE1*. However, SDE1 induced phenotypes were not observed in plants by co-infiltrated with *mSECP8* (Figures 3A,B,D). Additionally, no SDE1-associated phenotypes appeared on eGFP or mSECP8 single infiltration plants (Supplementary Figures 2A,B). Therefore, these results indicate that PCD suppressor mSECP8 inhibits SDE1-mediated necrosis and dwarfing in *N. benthamiana*.

**mSECP8 enhances the susceptibility of transgenic citrus to both Candidatus Liberibacter asiaticus and Xanthomonas citri subsp. citri**

To evaluate the role of mSECP8 on CLas colonization, transgenic citrus plants were generated and six lines (OE-1, OE-4, OE-8, OE-15, OE-17, and OE-25) were verified by both RT-PCR and GUS histochemical staining assay (Figures 4A,B). By comparing to line OE-8, the three lines (OE-1, OE-4, and OE-17) with greater transcript abundance were graft inoculated with CLas for further susceptibility evaluation (Figure 4C). CLas titers in OE-1, OE-4, and OE-17 lines were higher than that of WT plants at 40 dpi, especially at 70 dpi (Figure 4D). Nevertheless, CLas titers in WT plants got close to that in
transgenic plants at 100 dpi (Figure 4D). From 70 to 145 dpi, CLas associated chlorosis or mottled yellow symptoms were observed on the leaves of transgenic plants, and these transgenic lines were significantly dwarfing compared with WT controls (Figures 4E–G), while transgenic treatments and WT control showed no phenotype differences without CLas inoculation (Supplementary Figures 3A–C). These results suggest that mSECP8 contributes to CLas early proliferation and symptom development in transgenic citrus plants.

To observe whether mSECP8 enhanced susceptibility of transgenic plants to other pathogens, transgenic citrus leaves were inoculated using Xcc. Compared to WT plant, the lesion size (LZ) increased by 147.3~289.6% (Supplementary Figure 4A), and the disease index (DI) increased from 22.9 to 53.2~81.8% (Supplementary Figure 4B), and significantly more colony-forming units (CFUs) were observed in transgenic plants (Supplementary Figure 4C). Furthermore, leaves of Xcc-inoculated mSECP8-overexpressing plants exhibited remarkably earlier and larger pustule eruptions than those of control plants (Supplementary Figure 4D).

The expression level of the defense-related genes are remarkably decreased in mSECP8-transgenic citrus

To further investigate how mSECP8 interferes with plant defense responses, the expression of 10 immunity-related genes were analyzed. The results showed that the expression level of all the SAR-related (CsPR1, CsPR2, CsPR3, and CsPR5) and PTI marker (CsFRK1, CsGST1,
mSECP8-transgenic Wanjincheng orange plants were more susceptible to CLas. (A–C) Identification of mSECP8-transgenic plants. M, DL 2,000 DNA marker; 1, 4, 8, 15, 17, and 25 represented different SECP8-transgenic lines; WT1 and WT2, wild type of Wanjincheng plants; +, plGN-mSECP8 plasmid. (A) A 195 bp fragment was detected by RT-PCR using plGN-mSECP8-F/R primer pairs (Supplementary Table 1), the actin gene of citrus sinensis (CsActin) was used as internal reference. (B) Leaves of transgenic plants were used for GUS staining, and the blue-stained indicated positive plants. (C) SECP8 relative expression level in OE-1, OE-4, and OE-15, OE-17, and OE-25 compared with that in OE-8 was determined by RT-qPCR, the CsActin gene was used as internal reference. The asterisks indicate significant difference (**p < 0.01, ****p < 0.0001, determined by one-way ANOVA with Dunnett’s test). (D) The CLas titers in the mSECP8-transgenic citrus and WT control citrus were determined by qPCR at 0, 40, 70, and 100 dpi with CLas, the cytochrome oxidase gene of citrus sinensis (CsCOX) was chosen as internal

**FIGURE 4**

Continued
FIGURE 4 (Continued) control. Each Ct value was represented by means ± SE (nOE−mSECP8 = 3, nWT = 3). Asterisks represent significant differences in the Clas titer of 0, 40, 70, and 100 dpi between mSECP8-transgenic citrus and WT control by two-way ANOVA with Tukey’s test (***P < 0.001, and ****P < 0.0001). (E) Plants phenotype obtained at 70 dpi with Clas via branch and budding grafting. (F) Top view of Clas-infected WT and transgenic Wanjincheng plants at 145 dpi, and (G) symptoms of single leave detached from the contemporaneous plants. Scale bar represents 1 cm.

FIGURE 5 mSECP8 down-regulated the expression of defense-related genes in transgenic citrus. Transcript level of CsFRK1, CsGST1, CsNPR1, CsPR1, CsPR2, CsPR3, CsPR5, CsWRKY22, CsWRKY29, and CsTGA were significantly lower in mSECP8-transgenic than that in WT Wanjincheng plants. The CsActin gene was used as an endogenous control. The asterisks indicate significant difference (*P < 0.05, **p < 0.01, ***P < 0.001 and ****P < 0.0001, one-way ANOVA with Dunnett’s test). The experiment comprised at least three independent biological replicates and three technical replicates.

CsWRKY22, and CsWRKY29) genes were significantly reduced in transgenic plants (Figure 5). After challenging with Clas, similar results were exhibited in OE-mSECP8 plants (Supplementary Figure 5), indicating that mSECP8 may suppress the plants defense responses through interrupting SAR- and PTI-mediated pathways.

Discussion

In the host-pathogen arms race, on one hand, to prevent the spread of pathogen, host plants defense against pathogens by PTI and ETI with HR-associated PCD; on the other hand, to invade the host plants successfully, pathogens including bacteria, fungi and oomycetes evolve various effectors as virulence factors to suppress PTI or even ETI (Nimchuk et al., 2003; Jones and Dangl, 2006; Coll et al., 2011; Nguyen et al., 2021; Yuan et al., 2021). HR suppression of plant immune system plays a leading role in pathogenic virulence strategy. As is well known, numerous pathogens including bacteria, fungi, and oomycetes colonize extracellularly and suppress host HR by T3SS-secreted or Sec pathway-dependent effectors (Boller and Felix, 2009; Segers and Anné, 2011; Rocafort et al., 2020; Yuan et al., 2021).

PCD plays critical roles in plant immunity upon pathogen infection at early stage (Coll et al., 2011). By contrast, the pathogens secrete effectors to suppress cell death to facilitate their successful infection. Clas encodes a large number of Sec-dependent secretory proteins with unknown functions and a few have been characterized, but with only three, SDE1, m460, and AGH17470 identified as PCD elicitors, while others functioning as suppressors (Pitino et al., 2016, 2018; Liu et al., 2019; Du J. et al., 2022). In our study, mSECP8 was also identified as a suppressor by suppressing BAX- and INF-triggered PCD syndromes. It is noteworthy that mSECP8 interfered with SDE1-mediated water-soaked necrosis, the plant dwarfing and flowering delay (data not shown) in N. benthamiana. Previously, Clas effector SDE15 was verified as a broad-spectrum suppressor to inhibit PCD elicited by XccA* and Xanthomonas vesicatoria effector AvrBsT (Pang et al., 2020). Both SDE1 and mSECP8 play critical roles in citrus host and potentially target multiple host proteins due to their higher mRNA transcriptional levels in citrus than in psyllids with diverse subcellular localization. SDE1 is systematically distributed and has been demonstrated to interact with host PLCP proteins in reducing target activity to promote Clas infection (Clark et al., 2018). Therefore, mSECP8 is speculated as a potential broad-spectrum suppressor
of plant immunity, and potentially interacting with CLas elicitor SDE1 to co-manipulate plant immune responses. The counteractive effect of the two effectors could assist CLas escaping from host immunity, resulting in long latency and slowly developed symptoms of HLB. However, the molecular mechanisms of how mSECP8 interfering with SDE1 remains to be determined.

Systemic acquired resistance is highly associated with plant defense against bacterial pathogens (Durrant and Dong, 2004). In CLas-citrus interaction system, the expression of SAR-related genes (CsPR1, CsPR2, CsPR3, and CsPR5) and PTI marker genes (CsFRK1, CsGST1, CsWRKY22, and CsWRKY29) were remarkably down-regulated in transgenic plants expressing SDE15 by challenged with Xcc\textsuperscript{w}. Similarly, the transcript abundance of WRKY and PR genes was reduced in mSDE115-overexpressing citrus (Pang et al., 2020; Du M. et al., 2022). Whereas the expression of PR- and SA-related genes were significantly induced in AGH17470-transgenic citrus (Du J. et al., 2022). Intriguingly, 10 non-classic SecPs triggered the overexpression of NbPR1, NbPR2, and NbPR5 genes (Du et al., 2021). Herein, mSECP8 potentially promoted CLas infection via repression of SAR- and PTI-mediated defenses, but the detailed mechanisms of how mSECP8 suppress plant immunity remains to be addressed through finding its host interacting targets.

Conclusively, we identified the CLas-secreted Sec-dependent effector SECP8 as a broad-spectrum suppressor by inhibiting elicitor-triggered PCD in N. benthamiana and SDE1-induced phenotypes, and thereby provide the first evidence of counteractive effect of CLas suppressors and elicitors. Through susceptibility assessment of transgenic plants to CLas and Xcc, it revealed that mSECP8 may be involved in suppression of the plant immunity through manipulating SAR- and PTI-mediated defenses to facilitate pathogen infection. The present findings illustrate the role of SECP8 in pathogenicity of CLas and may shed new insight in CLas-citrus interactions.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary material.

Author contributions

PS, XW, and CZ designed the experiments. PS and XL performed the experiments. PS analyzed the data and wrote the manuscript. SF, XW, and CZ revised and polished the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2022.997825/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Bioinformatics features of SECP8. (A) The nucleotide sequence alignments among SECP8 (CLIBASIA_05330) and the homologies of other CLas typical strains from United States, China, and Japan. (B) SP and (C) transmembrane domain prediction of SECP8.

SUPPLEMENTARY FIGURE 2
No plant phenotype differences were induced by eGFP or mSECP8. (A,B) Photos were taken at 15 dpi, and scale bar represents 2 cm in (A).
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