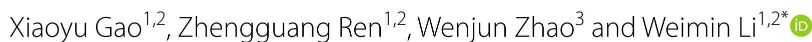
Phytopathology Research

RESEARCH Open Access

Candidatus Phytoplasma ziziphi encodes non-classically secreted proteins that suppress hypersensitive cell death response in *Nicotiana benthamiana*

Xiaoyu Gao[1,2], Zhengguang Ren[1,2], Wenjun Zhao[3] and Weimin Li[1,2*]



Abstract

Increasing evidence is proving the biological significance of the phytoplasma-secreted proteins. However, besides a few Sec-dependent secretory proteins, no other phytoplasma-secreted proteins have been reported yet. *Candidatus* Phytoplasma ziziphi is a phytoplasma that causes witches'-broom, a devastating jujube disease prevalent in east Asia. In this study, using the SecretomeP server coupled with an *Escherichia coli*-based alkaline phosphatase assay, we identified 25 non-classically secreted proteins (ncSecPs) from *Ca*. P. ziziphi, a novel type of secreted protein associated with phytoplasmas. Among them, six were characterized as hypersensitive cell death response (HR) suppressors that significantly attenuated both Bax- and INF1-triggered HR and H_2O_2 accumulation in *Nicotiana benthamiana*, indicating a so-far unknown role of the phytoplasma-secreted proteins. Further, we demonstrated that despite the diverse subcellular localizations in the *N. benthamiana* cells, the six HR-suppressing ncSecPs enhanced the gene expression of several known cell death inhibitors, including pathogenesis-related proteins (NbPR-1, NbPR-2, and NbPR-5) and Bax inhibitor-1 (NbBI-1 and NbBI-2). Together, our data indicated that *Ca*. P. ziziphi has evolved an arsenal of ncSecPs that jointly circumvent HR by activating the plant cell death inhibitors, thus providing new insight into understanding the pathogenesis of phytoplasmas.

Keywords *Candidatus* Phytoplasma ziziphi, Phytoplasma, Non-classically secreted protein, Hypersensitive response, Cell death inhibitor

Background

Phytoplasmas, belonging to the class Mollicutes, phylum Tenericutes, comprise a group of highly diverse phytopathogenic bacteria characterized by pleiomorphism, lack of a cell wall, and small genome size (Gundersen et al. 1994; Bertaccini 2007; Hogenhout et al. 2008; Pagliari and Musetti 2019; Bertaccini et al. 2022;). Under natural conditions, they are transmitted by dodder species and phloem-feeding insects, including leafhoppers, plant hoppers, and psyllids (Bertaccini 2007; Hogenhout et al. 2008). Phytoplasmas cause considerable damage worldwide in over 1,000 plant species, ranging from crops to fruit trees to ornamentals, with the infected plants usually displaying severe symptoms, including phyllody, virescence, the proliferation of shoots (Witches' broom), stunting, little leaf, and yellowing (Lee et al. 2000; Bertaccini 2007; Ermacora and Osler 2019).

*Correspondence:
Weimin Li
weimin.li@bua.edu.cn
[1] Key Laboratory for Northern Urban Agriculture of Ministry of Agriculture and Rural Affairs, Beijing University of Agriculture, Beijing 102206, China
[2] Department of Plant Protection, Beijing University of Agriculture, Beijing 102206, China
[3] CAIQ Center for Biosafety, Sanya 572024, China

© The Author(s) 2023. **Open Access** This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit http://creativecommons.org/licenses/by/4.0/.

To establish and facilitate infection of their hosts, the plant pathogenic bacteria usually utilize sophisticated export mechanisms to deliver so-called effector proteins into the host cells (Stavrinides et al. 2008; Deslandes and Rivas 2012). Since phytoplasmas have evolved from gram-positive ancestors (Weisburg et al. 1989), they lack the type III secretion system (T3SS), T4SS, and T6SS, which many Gram-negative bacteria usually used for delivering effectors into the host cells (Cambronne and Roy 2006; Shames and Finlay 2012; Galán and Waksman 2018). Instead, phytoplasmas harbor SecA, SecE, and SecY (Kakizawa et al. 2001, 2004; Barbara et al. 2002), the minimal set of components required for a general secretory (Sec) pathway that is conserved across all domains of life (Tsirigotaki et al. 2017), indicating that these bacteria export proteins through the Sec-dependent secretion system. Furthermore, mining of the genomes of phytoplasmas, like aster yellows phytoplasma strain witches' broom (AY-WB), onion yellows phytoplasma mild strain (OY-M), and peanut witches' broom phytoplasma (PnWB), revealed that all the phytoplasma species encode numerous proteins bearing a classical tripartite structured Sec signal peptide (SP), which guides the proteins into the bacterial extracellular space via the Sec pathway (Bai et al. 2009; Hoshi et al. 2009; Anabestani et al. 2017).

Among the phytoplasma Sec-dependent secretory proteins, a few have reportedly contributed to pathogenesis. For example, the AY-WB SAP54 and its homolog PHYL1 in OY-W phytoplasma mimic ubiquitin as a mediator between the MADS-domain transcription factors (MTFs) and the proteasome shuttle proteins RAD23 to promote the degradation of MTFs, which finally causes phyllody (MacLean et al. 2011, 2014; Maejima et al. 2014; Kitazawa et al. 2022). Additionally, AY-WB SAP11, as well as its homologs in wheat blue dwarf phytoplasma, apple proliferation phytoplasma and maize bushy stunt phytoplasma, induces shoot proliferation by interacting and destabilizing the TEOSINTE BRANCHED 1-CYCLOIDEA-PROLIFERATING CELL FACTOR (TCP) transcription factors (Sugio et al. 2011; Janik et al. 2017; Chang et al. 2018; Wang et al. 2018b; Pecher et al. 2019; Zhou et al. 2021). Moreover, a Sec-dependent protein of OY-M, namely TENGU, was reported to induce symptoms of witches' broom and dwarfism by inhibiting auxin-related pathways (Hoshi et al. 2009; Sugawara et al. 2013). Interestingly, besides interfering with plant development, SAP11 and SAP54 have been shown to either enhance insect vector reproduction or their colonization in plants (Sugio et al. 2011; MacLean et al. 2014).

The identification and characterization of the Sec-dependent secretory proteins greatly advance the knowledge of phytoplasma-plant interactions. Unfortunately, besides the Sec-dependent proteins, none of the other types of secreted proteins have been discovered in phytoplasmas. Non-classically secreted proteins (ncSecPs) are a type of secreted proteins that lack SPs or translocation signals but are still exported to the extracellular environment (D'Costa and Boyle 2000; Madureira et al. 2007; Pasztor et al. 2010; Oliveira et al. 2012; Ebner et al. 2016). Although the ncSecPs secretion mechanisms remain elusive, emerging evidence indicates that these proteins widely exist in bacteria and are vital to bacterial virulence or niche adaptation (Madureira et al. 2007; Pasztor et al. 2010; Oliveira et al. 2012; Ebner et al. 2016; Du et al. 2021). In this study, we reported that *Candidatus* Phytoplasma ziziphi, a phytoplasma associated with jujube witches'-broom (JWB), has evolved ncSecPs. Using in silico prediction coupled with an *Escherichia coli*-based alkaline phosphatase (PhoA) assay (Liu et al. 2019; Du et al. 2021), we identified 25 ncSecPs encoded by the *Ca.* P. ziziphi genome. Among them, six ncSecPs were shown to block hypersensitive cell death response (HR) triggered by both the pro-apoptotic mouse protein Bax (Lacomme and Santa Cruz 1999) and the *Phytophthora infestans* elicitin INF1 (Kamoun et al. 1998) in *Nicotiana bentamiana*. To the best of our knowledge, the HR-suppressing effector has not yet been identified from phytoplasmas to date. These novel effectors may shed light on how phytoplasmas overwhelm the host immunity.

Results

In silico analysis of the ncSecP candidates in *Ca.* P. ziziphi

The SecretomeP 2.0 software package has been trained to predict ncSecPs in Gram-positive and Gram-negative bacteria with the corresponding default settings of either 'Gram-positive bacteria' or 'Gram-negative bacteria' (Bendtsen et al. 2005). Here we employed this server to screen ncSecPs in *Ca.* P. ziziphi on a genome-wide scale. However, phytoplasmas are well-known for being in neither the Gram-positive group nor the Gram-negative group due to their distinguished membrane compositions (Razin et al. 1998). To improve the reliability and accuracy of the SecretomeP server on the phytoplasma ncSecP prediction, our strategy involved separately evaluating the annotated proteins of *Ca.* P. ziziphi with the 'Gram-positive bacteria' and 'Gram-negative bacteria' prediction models, with only the proteins scoring above 0.5 in both pipelines being recognized as the ncSecP candidates. After removing the redundant genes, 320 and 103 proteins with a score above 0.5 were identified by the models of 'Gram-positive bacteria' and 'Gram-negative bacteria', respectively (Additional file 1: Table S1). Further comparison of these two datasets revealed 95 common proteins, which represent the ncSecP candidates of *Ca.* P. ziziphi (Fig. 1 and Additional file 2: Table S2). Based on these putative ncSecPs, we particularly targeted those

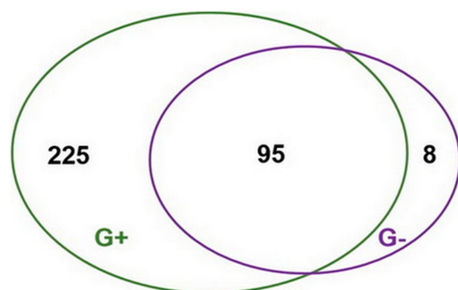


Fig. 1 Venn diagram of the *Candidatus* Phytoplasma ziziphi ncSecP candidates generated with the pipelines of 'Gram-positive bacteria' and 'Gram-negative bacteria' of the SecretomeP 2.0 server. 'G-' and 'G + ' represent the 'Gram-positive bacteria' and 'Gram-negative bacteria' models of SecretomeP 2.0, respectively

annotated as 'hypothetical protein' in an effort to identify novel proteins potentially involved in *Ca*. P. ziziphi virulence. Finally, a total of 37 related genes (Table 1) were successfully cloned and subjected to subsequent experiments.

Experimental verification of the ncSecP candidates of *Ca.* P. ziziphi

We further employed the *E.* coli-based PhoA system, a valid and reliable method for determining the bacterial ncSecPs (Du et al. 2021), to examine the secretion of the in silico predicted ncSecPs of *Ca*. P. ziziphi. We cloned the coding sequence of each selected ncSecP candidate (Table 1) into pET-mphoA (Fig. 2a), where it was fused with *mphoA* lacking a native SP sequence, to generate the pET-ncSecP-mphoA construct for the PhoA assay. The results showed that 12 among the 37 tested ncSecP candidates failed to be secreted (Table 1), as the bacterial cells expressing their mphoA fusion proteins showed no color change even after the 24 h incubation on indicator LB agar, similar to the negative control cells expressing only mphoA (Fig. 2b). In contrast, the mphoA fusion proteins of the other 25 ncSecP candidates unambiguously exhibited PhoA activity, and drove the bacterial cells to turn blue after between 12 and 24 h of incubation (Table 1and Fig. 2b). Therefore, these 25 ncSecP candidates were considered to be secreted and referred hereafter as Pzi-ncSecP.

Identification of Pzi-ncSecPs that suppress HR in *Nicotiana benthamiana*

When challenged with phytopathogens, plants may initiate HR, an active plant immune response accompanied by rapid and localized cell death (Coll et al. 2011; Stael et al. 2015). On the other hand, to counter this and ensure their success in plant infection, many pathogens evolve effector proteins that interfere with or suppress HR (Da Cunha et al. 2007; Dou and Zhou 2012; Lo Presti et al. 2015; Zvereva et al. 2016). However, whether *Ca.* P. ziziphi has evolved such a type of effector is still unknown. To clarify this issue, we employed a potato virus X (PVX)-based expression system coupled with two distinct HR inducers (Jones et al. 1999), the mouse pro-apoptotic mouse protein Bax and the *P. infestans* elicitin protein INF1 (Kamoun et al. 1998; Lacomme and Santa Cruz 1999), to identify the *Ca.* P. ziziphi effector(s) involved in HR suppression. Based on the assays, six of the 25 Pzi-ncSecPs, ncSecP 3, 9, 12, 14, 16, and 22, remarkably suppressed both Bax- and INF1-triggered HR in *N. benthamiana* (Fig. 3a, b). Moreover, consistent with their roles in HR suppression, these six Pzi-ncSecPs also inhibited the Bax- and INF1-triggered accumulation of H_2O_2 (Fig. 3c and Additional file 3: Figure S1), a critical reactive oxygen species (ROS) that contributes to HR (Petrov and Van Breusegem 2012). In contrast, the rest of the Pzi-ncSecPs inhibited neither cell death nor H_2O_2 accumulation triggered by Bax or INF1 (data not shown). Collectively, a total of six Pzi-ncSecPs were identified as HR suppressors, thus indicating a novel role of the phytoplasmal effectors.

Subcellular localization of the HR-suppressing Pzi-ncSecPs in *N. benthamiana*

We next examined the subcellular localizations of the six HR-suppressing Pzi-ncSecPs in plant cells. We fused the coding sequence of each Pzi-ncSecP with green fluorescence protein (GFP) reporter gene (Fig. 4a), and transiently expressed them in leaves of 4-week-old *N. benthamiana* leaves, followed by microscopic evaluation (Fig. 4b). The results indicated that ncSecP12 and ncSecP16, resembling free GFP, were evenly distributed in the cells. Although ncSecP9 was also present in the whole cell, it was less abundant in the nucleus than in the free GFP. Both ncSecP3 and ncSecP22 were predominately nuclear localized, and particularly, the latter was inclined to target the nucleolus. In addition, ncSecP14 was generally localized in both the cytoplasmic and the nuclear membrane but not the nucleus. Therefore, this suggested that the six Pzi-ncSecPs, after entering the plant cells, might target the different cellular compartments, thereby showing diverse subcellular localizations.

The HR-suppressing Pzi-ncSecPs enhanced gene expression of the cell death suppressors in *N. benthamiana*

To probe the HR suppression mechanisms of the six Pzi-ncSecPs, we investigated the expression of the defense-related genes in the *N. benthamiana* leaves that initially transiently expressed each of the HR-suppressing Pzi-ncSecPs. These were then challenged with Bax or INF1, with the leaves co-expressing GFP and Bax/INF1 being

Table 1 The *Candidatus* Phytoplasma ziziphi ncSecP candidates subjected to PhoA assays

Name	Protein ID	Length (aa)[a]	Annotation	PhoA activity[b]
ncSecP1	WP_121463741.1	217	hypothetical protein	+
ncSecP2	WP_121463765.1	146	hypothetical protein	++
ncSecP3	WP_161554967.1	52	hypothetical protein	++
ncSecP5	WP_121463805.1	123	hypothetical protein	++
ncSecP6	WP_121463806.1	286	hypothetical protein	++
ncSecP7	WP_121463824.1	277	hypothetical protein	+++
ncSecP8	WP_121463835.1	191	hypothetical protein	–
ncSecP9	WP_121463838.1	59	hypothetical protein	+
ncSecP10	WP_121463872.1	190	hypothetical protein	+
ncSecP11	WP_152031101.1	141	hypothetical protein	+
ncSecP12	WP_161554974.1	58	hypothetical protein	++
ncSecP13	WP_121463892.1	163	hypothetical protein	+
ncSecP14	WP_121463915.1	146	hypothetical protein	++
ncSecP15	WP_121463926.1	206	hypothetical protein	++
ncSecP16	WP_161554978.1	50	hypothetical protein	++
ncSecP18	WP_121463966.1	124	hypothetical protein	+
ncSecP19	WP_121463967.1	97	hypothetical protein	+
ncSecP20	WP_121463969.1	113	hypothetical protein	–
ncSecP21	WP_121463975.1	191	hypothetical protein	+++
ncSecP22	WP_121463976.1	196	hypothetical protein	++
ncSecP23	WP_121464013.1	105	hypothetical protein	–
ncSecP26	WP_121464021.1	163	hypothetical protein	+
ncSecP27	WP_121464077.1	211	hypothetical protein	–
ncSecP28	WP_152031104.1	90	hypothetical protein	–
ncSecP29	WP_121464083.1	153	hypothetical protein	+
ncSecP30	WP_121464103.1	228	hypothetical protein	–
ncSecP31	WP_121464104.1	148	hypothetical protein	+
ncSecP32	WP_152031107.1	65	hypothetical protein	–
ncSecP33	WP_121464189.1	75	hypothetical protein	+
ncSecP34	WP_161554994.1	53	hypothetical protein	–
ncSecP35	WP_121464264.1	107	hypothetical protein	+
ncSecP36	WP_121464202.1	278	hypothetical protein	++
ncSecP38	WP_121464213.1	222	hypothetical protein	+++

The protein IDs and annotation are based on the complete genomic data of *Ca.* P. ziziphi (GenBank No. CP025121.1)

[a] 'aa' indicates the number of amino acid residues of the protein

[b] PhoA assays were performed as described in the 'Methods' section. Single (+), double (++), and triple asterisks (+++) indicate weak, moderate, and strong PhoA activity, respectively. (–) indicates no phoA activity

the controls. Bax inhibitor-1 (BI-1) is a well-known cell death inhibitor in both plants and animals (Hückelhoven et al. 2003; Ishikawa et al. 2011). Using reverse transcription-quantitative PCR (RT-qPCR), we detected that the elevated transcriptional levels of both the *BI-1* homologous genes in *N. benthamiana* (*NbBI-1* and *NbBI-2*) in leaves expressing each of the Pzi-ncSecPs coupled with Bax or INF1 (Fig. 5a). Notably, the *NbBI-2* expression level was more significantly upregulated as compared with that of *NbBI-1* in any of the treated leaves, besides those co-expressing ncSecP3 and Bax, or ncSecP12 and INF1, in which the two *BI-1* genes exhibited a comparable increased level.

PR proteins (PR-1, PR-2, and PR-5) are the key defense components that positively promote basal resistance and systematic acquired resistance (SAR) in plants (Breen et al. 2017; Ali et al. 2018). However, recent studies showed that overexpression of the *PR* genes, in particular *PR-1* and *PR-5*, greatly inhibit HR (Lincoln et al. 2018; Du et al. 2021). We, therefore, evaluated whether the six HR-suppressing Pzi-ncSecPs interfered with the expression of *PR-1*, *PR-2*, and *PR-5*

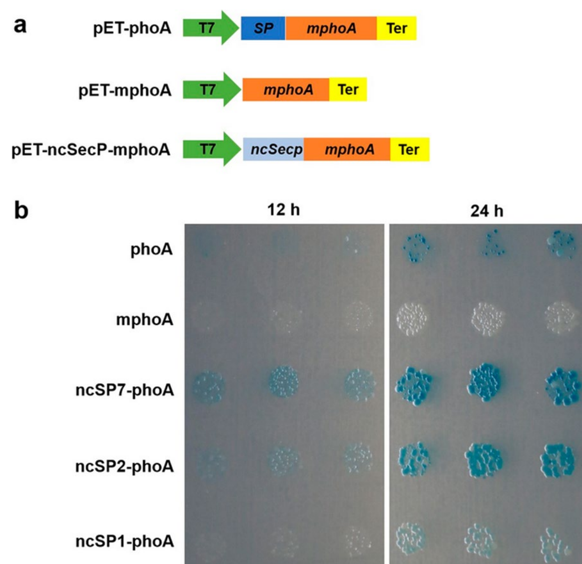


Fig. 2 Validation of the putative ncSecPs of *Candidatus* Phytoplasma ziziphi by using the *Escherichia coli*-based PhoA assays. **a** Diagrams of prokaryotic expression cassettes for *phoA* and its related fusion genes. The pET-phoA harboring the full-length *phoA* gene was used as the positive control, while pET-mphoA containing *mphoA* without a signal peptide (SP)-encoding sequence was used as the negative control. The *Ca.* P. ziziphin *ncSecP* was cloned into pET-mphoA and fused with *mphoA*, resulting in pET-ncSecP-mphoA. T7 indicates T7 promoter, and Ter represents T7 terminator. **b** PhoA assays verified the secretion of the in silico predicted *Ca.* P. ziziphin ncSecPs. The *E. coli* cells expressing phoA fusions were grown on the indicator LB medium, as described in the 'Methods' section. The images were taken after 12 and 24 h of incubation. Twenty-five of 37 candidates were clarified to be ncSecPs. Representatives of the ncSecPs with strong (ncSecP7), medium (ncSecP2), and weak (ncSecP1) secretion activities are shown

in *N. benthamiana*. The results showed that, in leaves expressing any of the six Pzi-ncSecPs together with Bax or INF1, the expression levels of the three *NbPR* genes were all substantially upregulated (Fig. 5b). Remarkably, the expression level of either *NbPR-1* or *NbPR-5* was more significantly upregulated than that of *NbPR-2* in the treated leaves, except the ncSecP 12- or ncSecP14-expressing leaves coupled with Bax, wherein the increased *NbPR-1* expression was lower than that of *NbPR-2*.

Discussion

While increasing numbers of ncSecPs have been identified in both Gram-positive and Gram-negative bacteria (D'Costa and Boyle 2000; Madureira et al. 2007; Pasztor et al. 2010; Oliveira et al. 2012; Ebner et al. 2016), these were never identified in phytoplasmas, a group of fastidious phloem-limited bacteria (Hogenhout et al. 2008; Pagliari and Musetti 2019). *Ca.* P. ziziphi is a phytoplasma with a highly reduced genome (only 0.75 M) encoding 694 putative proteins (Wang et al. 2018a). Here, using bioinformatics-based prediction coupled with the *E. coli*-based PhoA assay, we demonstrated that *Ca.* P. ziziphi encoded 25 ncSecPs (termed Pzi-ncSecPs), and thus proved that phytoplasmas have also evolved ncSecPs. Although numerous phytoplasma-secreted proteins have previously been identified, they all are Sec-dependent secretory proteins (Bai et al. 2009; Hoshi et al. 2009; Anabestani et al. 2017). Taken together, we propose that, except for the Sec-dependent secretion system, the phytoplasmas have evolved additional secretion apparatuses, like the non-classical secretion pathway(s), to deliver proteins into host plants.

When entering the host plants, the pathogen-secreted proteins usually target and usurp the host cell functions, including immune response, ubiquitination, protein modification, and cell signaling, thereby promoting infection and causing disease (Stavrinides et al. 2008; Deslandes and Rivas 2012). Likewise, in phytoplasmas, a few Sec-dependent secretory proteins have been uncovered to cause abnormal plant morphologies, including shoot proliferation and phyllody, by interfering with protein degradation or hormone signaling of the plants (Hoshi et al. 2009; Sugio et al. 2011; MacLean et al. 2011; Maejima et al. 2014; Janik et al. 2017; Wang et al. 2018b; Zhou et al. 2021), thus indicating their negative impacts on plant growth and development. However, the phytoplasmal effectors that counteract the plant immune responses remain unexplored. In this study, we showed that six of the 25 Pzi-ncSecPs were able to inhibit Bax- and INF1-triggered HR

(See figure on next page.)

Fig. 3 Effects of the *Candidatus* Phytoplasma ziziphi ncSecPs (Pzi-ncSecPs) on hypersensitive cell death response (HR) in *Nicotiana benthamiana*. **a** Six Pzi-ncSecPs inhibited the Bax- and INF1-triggered HR. A total of 25 Pzi-ncSecPs were examined with the HR suppression assays, as described in the Methods section, with the green fluorescent protein (GFP) as the negative control. HR symptoms were recorded five days post-*Bax*- or *INF1*-infiltration. The HR suppression activity of each Pzi-ncSecP was evaluated with the suppression number/infiltration number (CDS/I) index. The data from three independent experiments were combined as averages. Only the Pzi-ncSecPs exhibiting suppression (CDS/I $\geq$ 50%) are shown. Asterisks in columns indicate statistically significant differences based on Student's *t*-test (*, $P < 0.05$; **, $P < 0.01$). **b** HR suppression phenotypes caused by the Pzi-ncSecPs. Photos were taken five days after *Bax*- or *INF1*-infiltration. **c** Pzi-ncSecP16 suppressed Bax- and INF1-induced H_2O_2 accumulation. GFP was used as the negative control. Similar results were observed for Pzi-ncSecP3, 9, 12, 14, and 22 (Additional file 3: Figure S1). The *N. benthamiana* leaves were detached two days after *Bax*- or *INF1*-infiltration, and subsequently stained with DAB

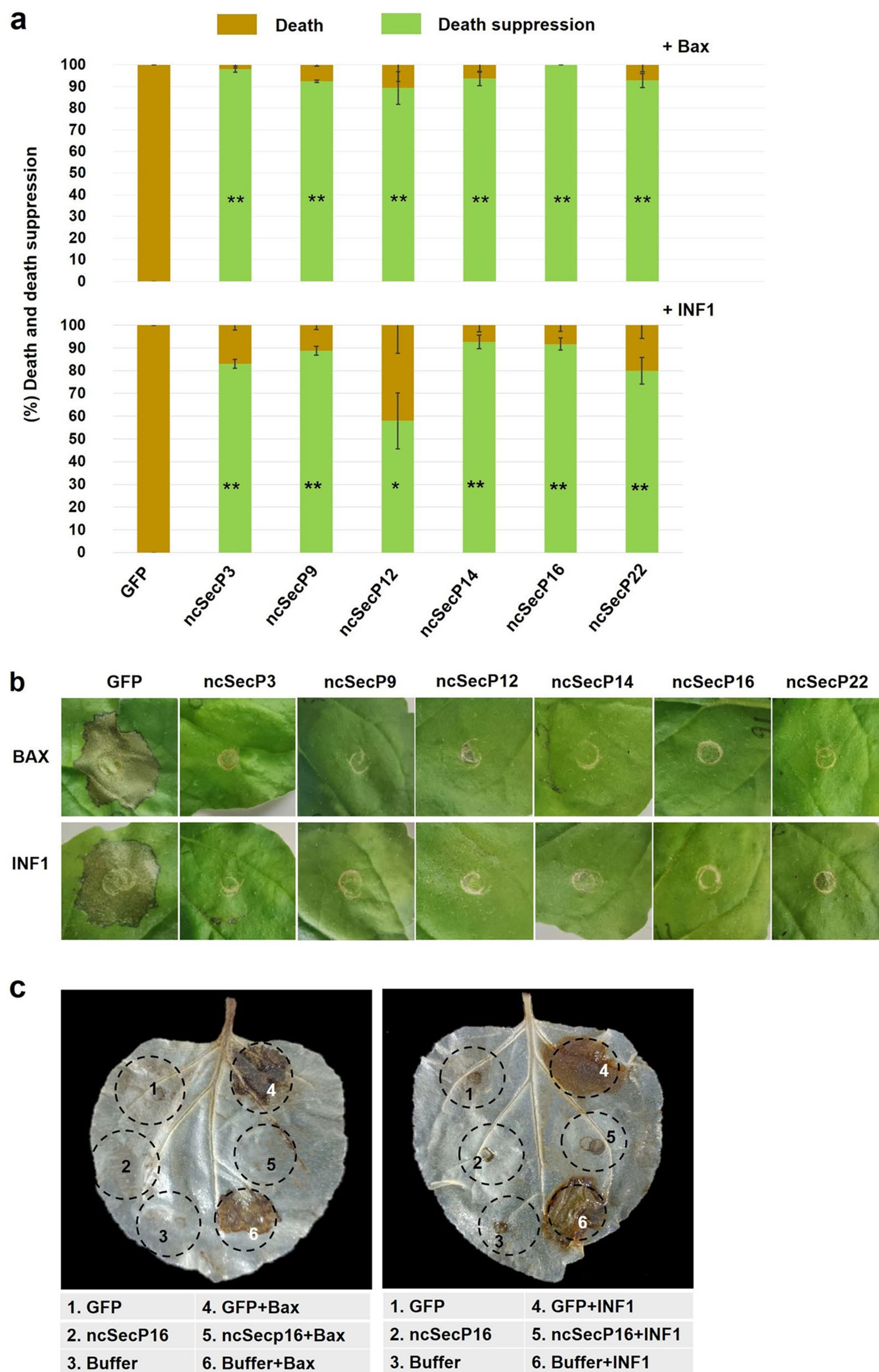


Fig. 3 (See legend on previous page.)

and H_2O_2 accumulation in *N. benthamiana*. HR represents a robust plant defense mechanism that restricts further colonization and the spread of plant pathogens at the infection site (Coll et al. 2011; Stael et al. 2015). Thus, the finding that the Pzi-ncSecPs acted as HR suppressors offers insights into how the phytoplasmas circumvent the plant immune responses.

The HR-suppressing effectors have been widely identified in both hemibiotrophs and biotrophs, including *Phytophthora* species (Wang et al. 2011; Pais et al. 2013) and *Pseudomonas syringae* (Jamir et al. 2004; Guo et al. 2009; Wei et al. 2018). Whereas many of them are known to suppress HR, usually by inactivating positive immune regulators (Jamir et al. 2004; Fujikawa et al. 2006; Rajput et al. 2014), emerging evidence indicates that some effectors instead are associated with negative regulators of HR. For example, the *P. infestans* RXLR effector Pi02860 suppresses HR by physically interacting with potato NPH3/RPT2-LIKE1 (NRL1), a predicted CULLIN3-associated ubiquitin E3 ligase that acts as a negative regulator of HR-induced cell death (Yang et al. 2016). This study showed that six HR-suppressing ncSecPs of *Ca.* P. ziziphi significantly enhanced the transcriptional levels of three *PR* genes (*NbPR-1*, *NbPR-2*, and *NbPR-5*) and *BI-1* homologous genes (*NbBI-1* and *NbBI-2*) in *N. benthamiana*, thereby suggesting that the Pzi-ncSecPs attenuated HR by hijacking the host cell death inhibitors. In addition, given their diverse plant subcellular localization patterns, these regulated the transcription of *PR* and *BI-1* genes probably by targeting different host cellular factors. Nevertheless, the data highlighted a potential strategy used by the *Ca.* P. ziziphi ncSecPs to counteract the plant immune responses.

The finding that the HR-suppressing ncSecPs of *Ca.* P. ziziphi induced transcription of *PR* genes was consistent with our recent study, where the Gram-negative bacterium '*Candidatus* Liberibacter asiaticus' (CLas) produce a suit of ncSecPs that inhibit HR presumably by inducing overexpression of *PR-1*, *PR-2,* and *PR-5* genes (Du et al. 2021). Like *Ca.* P. ziziphi, CLas is also an intracellular bacterium, which resides within the phloem cells (Wang et al. 2017). This prompted us to ask whether suppressing HR via ncSecPs to activate the host PR proteins represents a prevalent mechanism among intracellular bacteria.

In summary, the study showed that *Ca.* P. ziziphi has evolved an array of ncSecPs. Among them are six effectors that inhibit HR, presumably by upregulating the gene expression of the cell death inhibitors, including PR-1, PR-2, PR-5, and BI-1 homologs. To the best of our knowledge, all phytoplasmas determined to date induce diverse symptoms in their host plants (Lee et al. 2000; Bertaccini 2007; Ermacora and Osler 2019), but without HR-induced cell death, which indicates that the bacteria have evolved novel mechanisms to counteract the plant HR. In this study, the identification of the Pzi-ncSecPs as HR suppressors indicated that the *Ca.* P. ziziphi bacteria deployed their ncSecPs to activate the host cell death inhibitors, thereby compromising the plant HR to facilitate the bacterial infection and colonization (Fig. 6). This indicated a novel plant-phytoplasma interaction, which merits further investigation.

Conclusions

In this study, we showed that *Ca.* P. ziziphi, a type phytoplasma, encoded multiple ncSecPs that have not been previously identified from the phytoplasma species. Furthermore, a few ncSecPs were determined as HR suppressors that inhibited the plant HR presumably via the host cell death inhibitors. The results not only indicate the role of ncSecPs in phytoplasmal colonization and infection of the host plants but also shed light on how phytoplasmas overwhelm the plant immune responses.

Methods

Bioinformatics analysis

The annotated proteins derived from the complete *Ca.* P. ziziphi genome (GenBank No. CP025121.1) were deposited into the SecretomeP 2.0 Server (Bendtsen et al. 2005), followed by analysis with the default settings of 'Gram-positive bacteria' or 'Gram-negative bacteria'. The proteins that scored > 0.5 in both these prediction models were identified as the ncSecP candidates.

PhoA assay

PhoA assays were performed as described previously (Liu et al. 2019). First, the coding sequence of each tested protein was amplified using gene-specific primers (Additional file 4: Table S3), and subsequently cloned into the *Nde* I/*Hin*d III double-digested pET-mphoA vector containing the *mphoA*gene without a native SP-coding sequence. The resulting constructs were individually transformed into the *E. coli* BL21 cells. The PhoA activities of the transformants were detected on indicator LB agar supplemented with 90 μg/mL 5-bromo-4-chloro-3-indolyl phosphate (BCIP), 100 mM

(See figure on next page.)

Fig. 4 Subcellular localization of the HR-suppressing Pzi-ncSecPs in *Nicotiana benthamiana*. **a** Schematic of the expression cassettes for the green fluorescent protein (GFP) gene and the fusion gene *ncSecP-GFP*. 35S and NOS represent CaMV 35S promoter and nopaline synthase terminator, respectively. **b** Fluorescence of the ncSecP-GFP fusion proteins in the epidermal cells of the *N. benthamiana* leaves. The *N. benthamiana* leaves were infiltrated with agrobacterial cells harboring the *GFP* gene or the *ncSecP-GFP* fusion gene. At 60 h post-inoculation, GFP fluorescence was observed using confocal microscopy, with H2B-dsRed as the nuclear marker. Scale bars indicate 20 μm

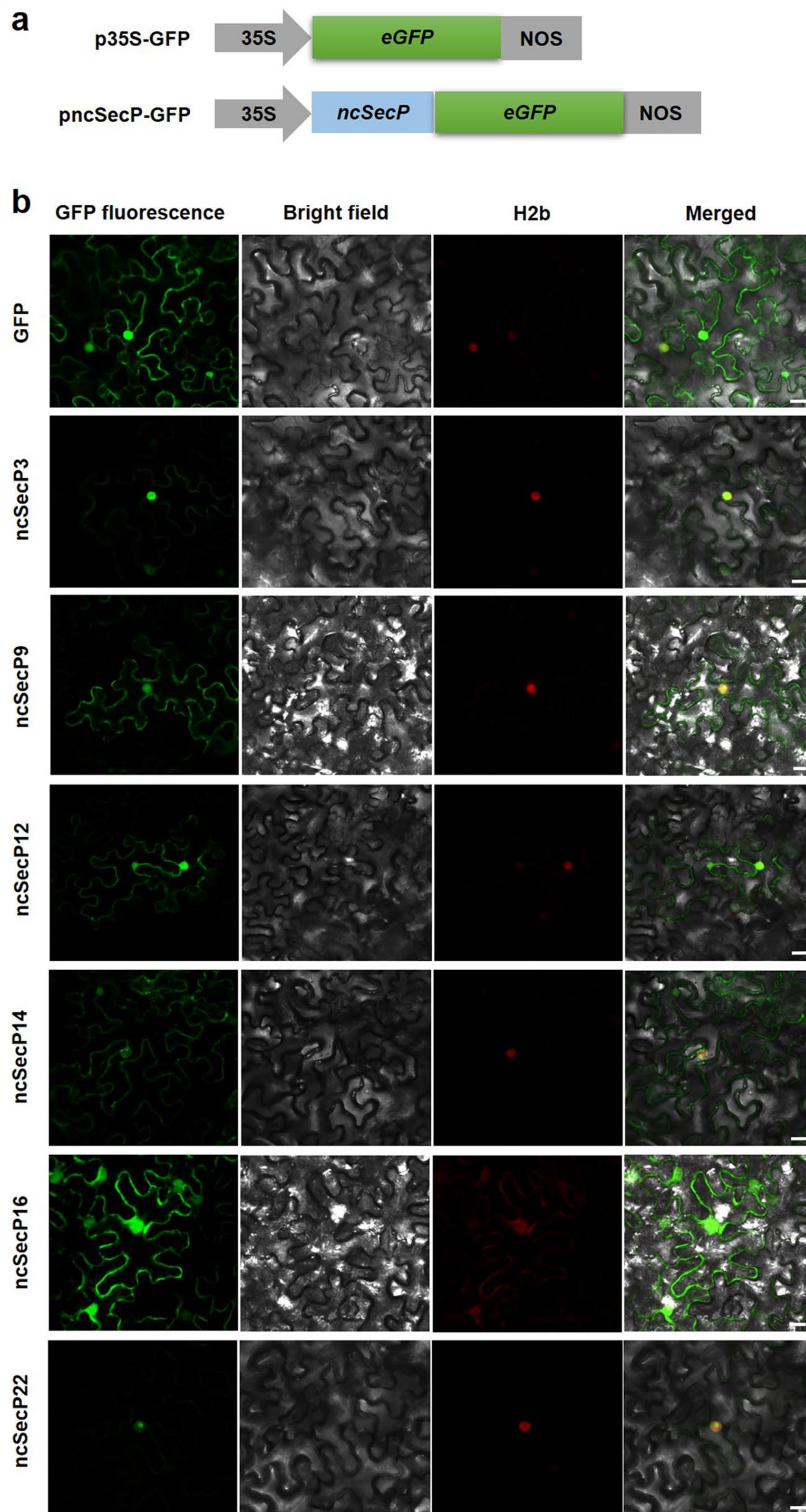


Fig. 4 (See legend on previous page.)

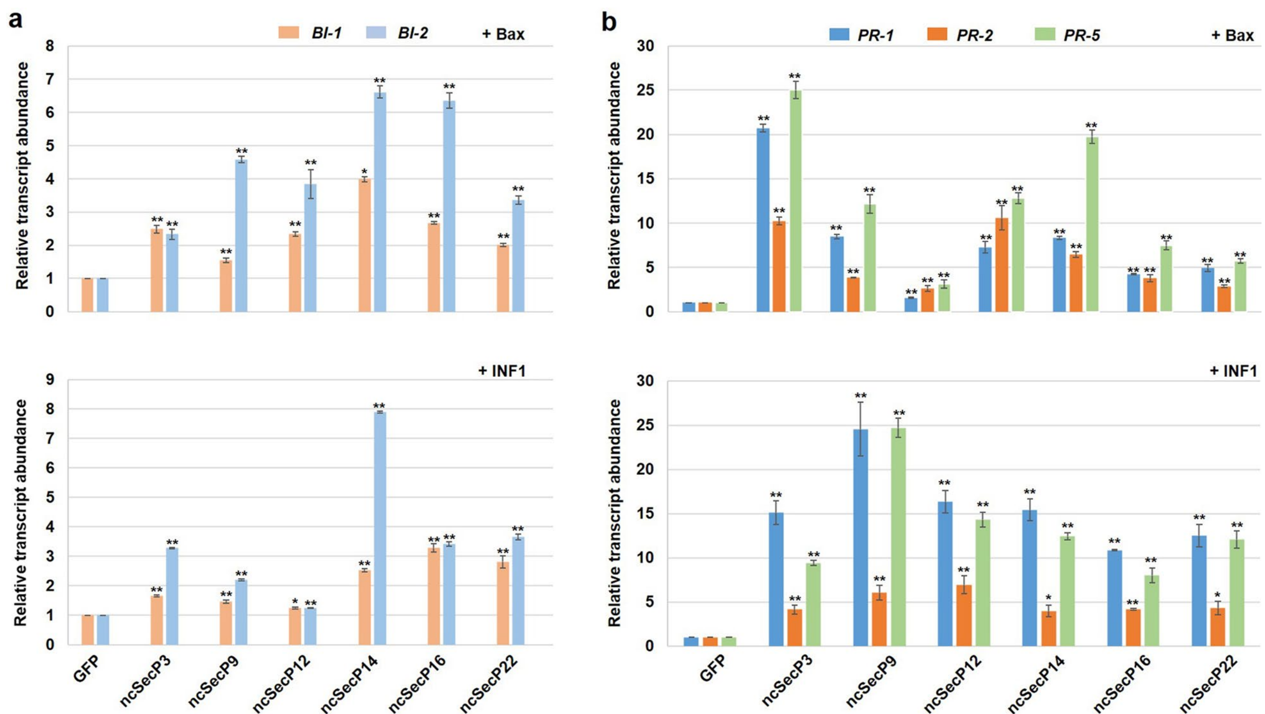


Fig. 5 The HR-suppressing Pzi-ncSecPs enhanced gene expression of cell death inhibitors in *Nicotiana benthamiana*. The *N. benthamiana* leaves were individually inoculated with the agrobacterial cells bearing the *Pzi-ncSecP* or the *GFP* genes, and further challenged with Bax or INF1 at 24 h post-inoculation (hpi). The leaves were harvested at 24 h post-*Bax* or *INF1* infiltration, followed by the total RNA extraction. The relative transcriptional levels of three pathogenesis-related genes (*NbPR-1*, *NbPR-2*, and *NbPR-5*) **(a)** and two Bax inhibitor-1 genes (*NbBI-1* and *NbBI-2*) **(b)** were analyzed by using RT-qPCR. Three biological replicates were performed, each with three technical replicates. The *N. benthamiana actin* gene was employed as the internal reference. Bars indicate the standard errors of the mean, and asterisks show statistically significant differences based on Student's *t*-tests (*, $P < 0.05$, **, $P < 0.01$)

isopropyl β-D-1-thiogalactopyranoside (IPTG), and 75 mM Na_2HPO_4] at 37°C after up to 24 h of incubation. The *E. coli* BL21 cells containing the empty pET-mphoA vector remained white after 24 h of incubation, and were used as the negative control, while those with the pET-phoA vector turned to blue after 6–12 h incubation, and were then used as the positive control.

HR suppression assay and 3, 3′-Diaminobenzidine (DAB) staining

The coding sequences of the selected ncSecPs were amplified with the primers listed in Additional file 4: Table S3, and individually ligated into the *Cla* I/*Sal* I-digested pGR107 vector, a binary plant expression vector based on PVX (Jones et al. 1999), resulting in the constructs pPVX-ncSecPn (in which 'n' represents an integer from 1 to 27). Subsequently, the constructs were transformed into the *Agrobacterium tumefaciens* strain GV3101, followed by HR suppression assays via agro-infiltration as described previously (Wang et al. 2011). First, the fourth and fifth leaves of the five 6-week-old *N. benthamiana* plants were initially infiltrated with the *A. tumefaciens* cells harboring the pPVX-ncSecPn. The *A. tumefaciens* cells carrying pPVX-GFP were used as a negative control. At 24 h post-inoculation (hpi), the infiltration sites were further inoculated with the cells containing pPVX-Bax or pPVX-INF1. At 48 hpi, some of the inoculated leaves were detached for DAB staining as previously described (Vanacker et al. 2000), and the remaining leaves were used to record cell death development for up to 5 days post inoculation (dpi). The experiment was repeated thrice. The HR-suppression ability of each ncSecP was evaluated with the cell death suppression number/infiltration number (CDS/I) index, which was recorded as either suppression (CDS/I ≥ 50%), or no suppression (CDS/I < 50%).

Confocal imaging

The coding sequences of the selected ncSecPs were amplified with the gene-specific primers (Additional file 4: Table S3), and were further individually cloned into the *Kpn* I/*Xho* I-digested pCAMBIA1300-35S-GFP vector, generating the constructs carrying a fusion gene *ncSecP-GFP*. The constructs were then transformed

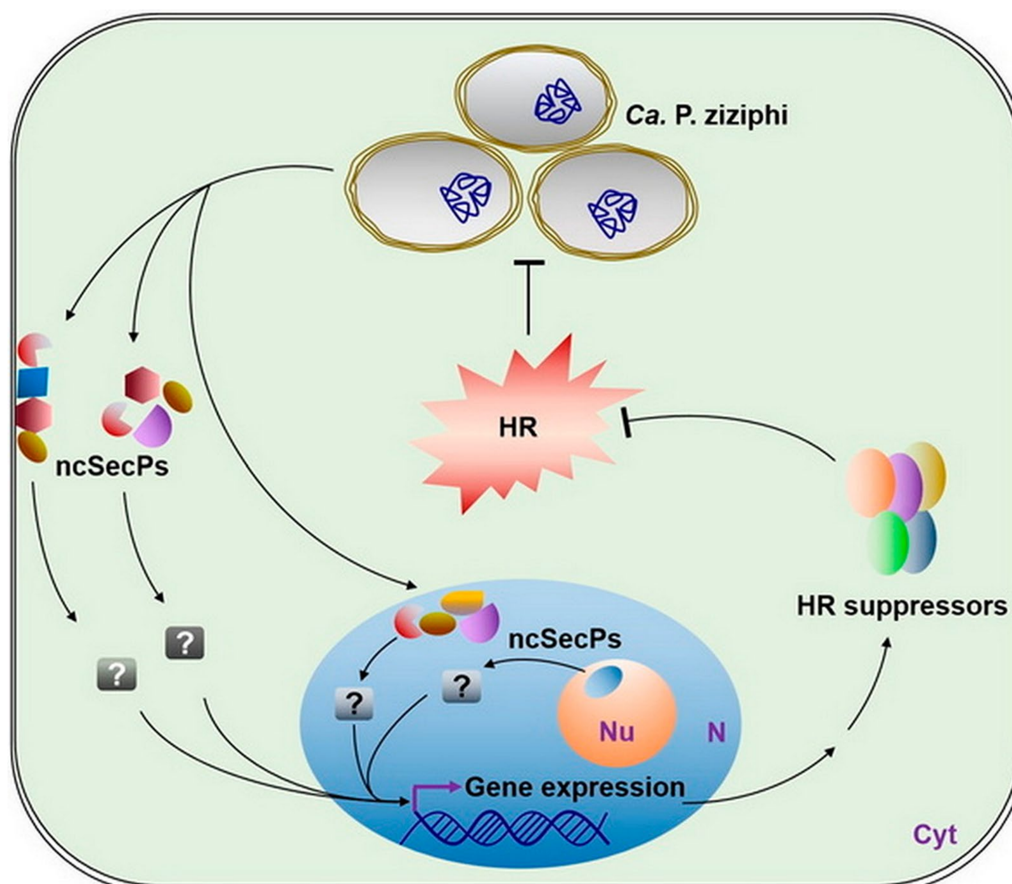


Fig. 6 The proposed model for the *Candidatus* Phytoplasma ziziphi bacterium deploying their ncSecPs to circumvent the host plant HR. As an intracellular bacterium, *Ca.* P. ziziphi secretes at least six ncSecPs that may target different plant subcellular compartments but work together to upregulate the gene expression of specific HR inhibitors, including pathogenesis-related proteins (PR-1, PR-2, and PR-5) and Bax inhibitor-1 (BI-1), via unknown mechanisms. The overexpressed HR inhibitors subsequently cause HR suppression, which favors colonization and infection of *Ca.* P. ziziphi. Blunt arrows indicate the negative impacts. 'Cyt' represents cytoplasm, while 'N' and 'Nu' indicate nucleus and nucleolus, respectively

into *A. tumefaciens* EHA105, followed by agroinfiltration of the 4-week-old *N. benthamiana* leaves. After 60 hpi, the infiltrated leaves were visualized with a TCS SP5 confocal microscope (Leica, Germany). GFP was excited at 488 nm, and the fluorescence emission was captured between 500 and 530 nm. Red fluorescent protein (DsRed) was excited at 543 nm, and the fluorescence emission was detected between 590 and 630 nm.

RT-qPCR analysis

The leaves of 6-week-old *N. benthamiana* plants were first infiltrated with the agrobacterial cells harboring the pPVX-ncSecPm (wherein 'm' represents 3, 9, 12, 14, 16, or 22) or pPVX-GFP. This was followed by the second infiltration with the *A. tumefaciens* cells carrying pPVX-Bax or pPVX-INF1 at 24 hpi, as described in the 'HR suppression assay and DAB staining' section. At 24 hpi of Bax or INF1, the infiltrated leaf tissues were collected to extract total RNA with the RNeasy Mini Kit (Qiagen, USA), followed by cDNA synthesis using random primers and a PrimeScript RT reagent kit with gDNA Eraser (TaKaRa, Japan). The resulting cDNA samples were then subjected to RT-qPCR analysis by using the TB Green Premix EX TaqII (TaKaRa, Japan) and gene-specific primers (Additional file 4: Table S3) on the ABI StepOnePlus™ Real-Time PCR instrument (Applied Biosystems, Foster City, CA), as previously described (Zhang et al. 2019). The *N. benthamiana* actin gene (Genbank No. JQ256516.1) was used as the internal reference gene. Each reaction was performed using three biological replicates (each containing three technical replicates). The relative gene expression values were calculated using the C_T method ($2^{-\Delta\Delta CT}$) and then converted to fold-change values (Livak and Schmittgen 2001). Then, statistical analysis of the data was performed using Student's *t*-test.

Abbreviations

AY-WB Aster yellows phytoplasma strain witches' broom
BI-1 Bax inhibitor-1
Ca. P. ziziphi *Candidatus* Phytoplasma ziziphi
Cyt Cytoplasm
dpi Days post inoculation
G- Gram-positive bacteria
G+ Gram-negative bacteria
GFP Green fluorescence protein
HR Hypersensitive response
JWB Jujube witches'-broom
MTF MADS-domain transcription factor
N Nucleus
ncSecP Non-classically secreted protein
NRL1 NPH3/RPT2-LIKE1
Nu Nucleolus
OY-M Onion yellows phytoplasma mild strain
PhoA Alkaline phosphatase
PnWB Peanut witches'broom phytoplasma
PR Pathogenesis-related
SAR Systematic acquired resistance
SP Signal peptide
T3SS Type III secretion system
TCP Teosinte branched 1-cycloidea-proliferating cell factor

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s42483-023-00166-0.

Additional file 1: Table S1. The *Candidatus* Phytoplasma ziziphi ncSecP candidates predicted with the 'Gram-positive bacteria' and 'Gram-negative bacteria' models of SecretomeP 2.0.

Additional file 2: Table S2. The common *Candidatus* Phytoplasma ziziphi ncSecP candidates generated from both 'Gram-positive bacteria' and 'Gram-negative bacteria' models of SecretomeP 2.0.

Additional file 3: Figure S1. Five non-classically secreted proteins (ncSecPs) of *Candidatus* Phytoplasma ziziphi suppressed H_2O_2 accumulation.

Additional file 4: Table S3. The primers used in this study.

Acknowledgements

We thank Ms. Shuang Wang (Chinese Academy of Agricultural Sciences) for her helpful assistance in the cloning of the *Candidatus* Phytoplasma ziziphi genes.

Authors' contributions

WL, ZR, and WZ designed the research. XG and WL performed the research. WL, ZR, WZ, and XG analyzed the data and wrote the manuscript. All authors read and approved the final manuscript.

Funding

This study was funded by the National Key Research and Development Program (2021YFC2600602), the National Natural Science Foundation of China (31970126), and the Science Innovation Program of Beijing University of Agriculture.

Availability of data and materials

Not applicable.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Received: 14 October 2022 Accepted: 7 February 2023
Published online: 28 March 2023

References

Ali S, Ganai BA, Kamili AN, Bhat AA, Mir ZA, Bhat JA, et al. Pathogenesis-related proteins and peptides as promising tools for engineering plants with multiple stress tolerance. Microbiol Res. 2018;212–213:29–37. https://doi.org/10.1016/j.micres.2018.04.008.

Anabestani A, Izadpanah K, Abbà S, Galetto L, Ghorbani A, Palmano S, et al. Identification of putative effector genes and their transcripts in three strains related to '*Candidatus* Phytoplasma aurantifolia'. Microbiol Res. 2017;199:57–66. https://doi.org/10.1016/j.micres.2017.03.001.

Bai X, Correa VR, Toruño TY, Ammar El D, Kamoun S, Hogenhout SA. AY-WB phytoplasma secretes a protein that targets plant cell nuclei. Mol Plant Microbe Interact. 2009;22:18–30. https://doi.org/10.1094/MPMI-22-1-0018.

Barbara DJ, Morton A, Clark MF, Davies DL. Immunodominant membrane proteins from two phytoplasmas in the aster yellows clade (chlorante aster yellows and clover phyllody) are highly divergent in the major hydrophilic region. Microbiology. 2002;148:157–67. https://doi.org/10.1099/00221287-148-1-157.

Bendtsen JD, Kiemer L, Fausbøll A, Brunak S. Non-classical protein secretion in bacteria. BMC Microbiol. 2005;5:58. https://doi.org/10.1186/1471-2180-5-58.

Bertaccini A, Arocha-Rosete Y, Contaldo N, Duduk B, Fiore N, Montano HG, et al. Revision of the '*Candidatus* Phytoplasma' species description guidelines. Int J Syst Evol Microbiol. 2022;72(4):10. https://doi.org/10.1099/ijsem.0.005353.

Bertaccini A. Phytoplasmas: diversity, taxonomy, and epidemiology. Front Biosci. 2007;12:673–89. https://doi.org/10.2741/2092.

Breen S, Williams SJ, Outram M, Kobe B, Solomon PS. Emerging insights into the functions of pathogenesis-related protein 1. Trends Plant Sci. 2017;22:871–9. https://doi.org/10.1016/j.tplants.2017.06.013.

Cambronne ED, Roy CR. Recognition and delivery of effector proteins into eukaryotic cells by bacterial secretion systems. Traffic. 2006;7:929–39. https://doi.org/10.1111/j.1600-0854.2006.00446.x.

Chang SH, Tan CM, Wu CT, Lin TH, Jiang SY, Liu RC, et al. Alterations of plant architecture and phase transition by the phytoplasma virulence factor SAP11. J Exp Bot. 2018;69:5389–401. https://doi.org/10.1093/jxb/ery318.

Coll NS, Epple P, Dangl JL. Programmed cell death in the plant immune system. Cell Death Differ. 2011;18:1247–56. https://doi.org/10.1038/cdd.2011.37.

Da Cunha L, Sreerekha MV, Mackey D. Defense suppression by virulence effectors of bacterial phytopathogens. Curr Opin Plant Biol. 2007;10:349–57. https://doi.org/10.1016/j.pbi.2007.04.018.

D'Costa SS, Boyle MD. Interaction of group A streptococci with human plasmin(ogen) under physiological conditions. Methods. 2000;21:165–77. https://doi.org/10.1006/meth.2000.0988.

Deslandes L, Rivas S. Catch me if you can: bacterial effectors and plant targets. Trends Plant Sci. 2012;17:644–55. https://doi.org/10.1016/j.tplants.2012.06.011.

Dou D, Zhou JM. Phytopathogen effectors subverting host immunity: different foes, similar battleground. Cell Host Microbe. 2012;12:484–95. https://doi.org/10.1016/j.chom.2012.09.003.

Du P, Zhang C, Zou X, Zhu Z, Yan H, Wuriyanghan H, et al. *Candidatus* Liberibacter asiaticus" secretes nonclassically secreted proteins that suppress host hypersensitive cell death and induce expression of plant pathogenesis-related proteins. Appl Environ Microbiol. 2021;87:e00019-21. https://doi.org/10.1128/AEM.00019-21.

Ebner P, Rinker J, Nguyen MT, Popella P, Nega M, Luqman A, et al. Excreted cytoplasmic proteins contribute to pathogenicity in *Staphylococcus aureus*. Infect Immun. 2016;84:1672–81. https://doi.org/10.1128/IAI.00138-16.

Ermacora P, Osler R. Symptoms of phytoplasma diseases. Methods Mol Biol. 2019;1875:53–67. https://doi.org/10.1007/978-1-4939-8837-2_5.

Fujikawa T, Ishihara H, Leach JE, Tsuyumu S. Suppression of defense response in plants by the *avrBs3/pthA* gene family of *Xanthomonas* spp. Mol Plant Microbe Interact. 2006;19:342–9. https://doi.org/10.1094/MPMI-19-0342.
Galán JE, Waksman G. Protein-injection machines in bacteria. Cell. 2018;172:1306–18. https://doi.org/10.1016/j.cell.2018.01.034.
Gundersen DE, Lee IM, Rehner SA, Davis RE, Kingsbury DT. Phylogeny of mycoplasmalike organisms (phytoplasmas): a basis for their classification. J Bacteriol. 1994;176:5244–54. https://doi.org/10.1128/jb.176.17.5244-5254.1994.
Guo M, Tian F, Wamboldt Y, Alfano JR. The majority of the type III effector inventory of Pseudomonas syringae pv tomato DC3000 can suppress plant immunity. Mol Plant Microbe Interact. 2009;22:1069–80. https://doi.org/10.1094/MPMI-22-9-1069.
Hogenhout SA, Oshima K, el Ammar D, Kakizawa S, Kingdom HN, Namba S. Phytoplasmas: bacteria that manipulate plants and insects. Mol Plant Pathol. 2008;9:403–23. https://doi.org/10.1111/j.1364-3703.2008.00472.x.
Hoshi A, Oshima K, Kakizawa S, Ishii Y, Ozeki J, Hashimoto M, et al. A unique virulence factor for proliferation and dwarfism in plants identified from a phytopathogenic bacterium. Proc Natl Acad Sci U S A. 2009;106:6416–21. https://doi.org/10.1073/pnas.0813038106.
Hückelhoven R, Dechert C, Kogel KH. Overexpression of barley BAX inhibitor 1 induces breakdown of mlo-mediated penetration resistance to *Blumeria graminis*. Proc Natl Acad Sci U S A. 2003;100:5555–60. https://doi.org/10.1073/pnas.0931464100.
Ishikawa T, Watanabe N, Nagano M, Kawai-Yamada M, Lam E. Bax inhibitor-1: a highly conserved endoplasmic reticulum-resident cell death suppressor. Cell Death Differ. 2011;18:1271–8. https://doi.org/10.1038/cdd.2011.59.
Jamir Y, Guo M, Oh HS, Petnicki-Ocwieja T, Chen S, Tang X, et al. Identification of *Pseudomonas syringae* type III effectors that can suppress programmed cell death in plants and yeast. Plant J. 2004;37:554–65. https://doi.org/10.1046/j.1365-313x.2003.01982.x.
Janik K, Mithöfer A, Raffeiner M, Stellmach H, Hause B, Schlink K. An effector of apple proliferation phytoplasma targets TCP transcription factors-a generalized virulence strategy of phytoplasma? Mol Plant Pathol. 2017;18:435–42. https://doi.org/10.1111/mpp.12409.
Jones L, Hamilton AJ, Voinnet O, Thomas CL, Maule AJ, Baulcombe DC. RNA-DNA interactions and DNA methylation in post-transcriptional gene silencing. Plant Cell. 1999;11:2291–301. https://doi.org/10.1105/tpc.11.12.2291.
Kakizawa S, Oshima K, Kuboyama T, Nishigawa H, Jung H, Sawayanagi T, et al. Cloning and expression analysis of Phytoplasma protein translocation genes. Mol Plant Microbe Interact. 2001;14:1043–50. https://doi.org/10.1094/MPMI.2001.14.9.1043.
Kakizawa S, Oshima K, Nishigawa H, Jung HY, Wei W, Suzuki S, et al. Secretion of immunodominant membrane protein from onion yellows phytoplasma through the Sec protein-translocation system in *Escherichia coli*. Microbiology. 2004;150:135–42. https://doi.org/10.1099/mic.0.26521-0.
Kamoun S, van West P, Vleeshouwers VG, de Groot KE, Govers F. Resistance of *Nicotiana benthamiana* to *Phytophthora infestans* is mediated by the recognition of the elicitor protein INF1. Plant Cell. 1998;10:1413–26. https://doi.org/10.1105/tpc.10.9.1413.
Kitazawa Y, Iwabuchi N, Maejima K, Sasano M, Matsumoto O, Koinuma H, et al. A phytoplasma effector acts as a ubiquitin-like mediator between floral MADS-box proteins and proteasome shuttle proteins. Plant Cell. 2022;34:1709–23. https://doi.org/10.1093/plcell/koac062.
Lacomme C, Santa CS. Bax-induced cell death in tobacco is similar to the hypersensitive response. Proc Natl Acad Sci U S A. 1999;96:7956–61. https://doi.org/10.1073/pnas.96.14.7956.
Lee IM, Davis RE, Gundersen-Rindal DE. Phytoplasma: phytopathogenic mollicutes. Annu Rev Microbiol. 2000;54:221–55. https://doi.org/10.1146/annurev.micro.54.1.221.
Lincoln JE, Sanchez JP, Zumstein K, Gilchrist DG. Plant and animal PR1 family members inhibit programmed cell death and suppress bacterial pathogens in plant tissues. Mol Plant Pathol. 2018;19:2111–23. https://doi.org/10.1111/mpp.12685.
Liu X, Fan Y, Zhang C, Dai M, Wang X, Li W. Nuclear Import of a secreted "*Candidatus* Liberibacter asiaticus" protein is temperature dependent and contributes to pathogenicity in *Nicotiana benthamiana*. Front Microbiol. 2019;10:1684. https://doi.org/10.3389/fmicb.2019.01684.
Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods (san Diego, Calif). 2001;25:402–8. https://doi.org/10.1006/meth.2001.1262.
Lo Presti L, Lanver D, Schweizer G, Tanaka S, Liang L, Tollot M, et al. Fungal effectors and plant susceptibility. Annu Rev Plant Biol. 2015;66:513–45. https://doi.org/10.1146/annurev-arplant-043014-114623.
MacLean AM, Orlovskis Z, Kowitwanich K, Zdziarska AM, Angenent GC, Immink RG, et al. Phytoplasma effector SAP54 hijacks plant reproduction by degrading MADS-box proteins and promotes insect colonization in a RAD23-dependent manner. PLoS Biol. 2014;12:1001835. https://doi.org/10.1371/journal.pbio.1001835.
MacLean AM, Sugio A, Makarova OV, Findlay KC, Grieve VM, Tóth R, et al. Phytoplasma effector SAP54 induces indeterminate leaf-like flower development in *Arabidopsis* plants. Plant Physiol. 2011;157:831–41. https://doi.org/10.1104/pp.111.181586.
Madureira P, Baptista M, Vieira M, Magalhães V, Camelo A, Oliveira L, et al. *Streptococcus agalactiae* GAPDH is a virulence-associated immunomodulatory protein. J Immun. 2007;178:1379–87. https://doi.org/10.4049/jimmunol.178.3.1379.
Maejima K, Iwai R, Himeno M, Komatsu K, Kitazawa Y, Fujita N, et al. Recognition of floral homeotic MADS domain transcription factors by a phytoplasmal effector, phyllogen, induces phyllody. Plant J. 2014;78:541–54. https://doi.org/10.1111/tpj.12495.
Oliveira L, Madureira P, Andrade EB, Bouaboud A, Morello E, Ferreira P, et al. Group B streptococcus GAPDH is released upon cell lysis, associates with bacterial surface, and induces apoptosis in murine macrophages. PLoS ONE. 2012;7:e29963. https://doi.org/10.1371/journal.pone.0029963.
Pagliari L, Musetti R. Phytoplasmas: an introduction. Methods Mol Biol. 2019;1875:1–6. https://doi.org/10.1007/978-1-4939-8837-2_1.
Pais M, Win J, Yoshida K, Etherington GJ, Cano LM, Raffaele S, et al. From pathogen genomes to host plant processes: the power of plant parasitic oomycetes. Genome Biol. 2013;14:211. https://doi.org/10.1186/gb-2013-14-6-211.
Pasztor L, Ziebandt AK, Nega M, Schlag M, Haase S, Franz-Wachtel M, et al. Staphylococcal major autolysin (Atl) is involved in excretion of cytoplasmic proteins. J Biol Chem. 2010;285:36794–803. https://doi.org/10.1074/jbc.M110.167312.
Pecher P, Moro G, Canale MC, Capdevielle S, Singh A, MacLean A, et al. Phytoplasma SAP11 effector destabilization of TCP transcription factors differentially impact development and defence of Arabidopsis versus maize. PLoS Pathog. 2019;15:1008035. https://doi.org/10.1371/journal.ppat.1008035.
Petrov VD, Van Breusegem F. Hydrogen peroxide-a central hub for information flow in plant cells. AoB Plants. 2012. https://doi.org/10.1093/aobpla/pls014.
Rajput NA, Zhang M, Ru Y, Liu T, Xu J, Liu L, et al. *Phytophthora sojae* effector PsCRN70 suppresses plant defenses in *Nicotiana benthamiana*. PLoS ONE. 2014;9:e98114. https://doi.org/10.1371/journal.pone.0098114.
Razin S, Yogev D, Naot Y. Molecular biology and pathogenicity of mycoplasmas. Microbiol Mol Biol Rev. 1998;62:1094–156. https://doi.org/10.1128/MMBR.62.4.1094-1156.1998.
Shames SR, Finlay BB. Bacterial effector interplay: a new way to view effector function. Trends Microbiol. 2012;20:214–9. https://doi.org/10.1016/j.tim.2012.02.007.
Stael S, Kmiecik P, Willems P, Van Der Kelen K, Coll NS, Teige M, et al. Plant innate immunity–sunny side up? Trends Plant Sci. 2015;20:3–11. https://doi.org/10.1016/j.tplants.2014.10.002.
Stavrinides J, McCann HC, Guttman DS. Host-pathogen interplay and the evolution of bacterial effectors. Cell Microbiol. 2008;10:285–92. https://doi.org/10.1111/j.1462-5822.2007.01078.x.
Sugio A, Kingdom HN, MacLean AM, Grieve VM, Hogenhout SA. Phytoplasma protein effector SAP11 enhances insect vector reproduction by manipulating plant development and defense hormone biosynthesis. Proc Natl Acad Sci U S A. 2011;108:E1254–63. https://doi.org/10.1073/pnas.1105664108.
Sugawara K, Honma Y, Komatsu K, Himeno M, Oshima K, Namba S. The alteration of plant morphology by small peptides released from the proteolytic processing of the bacterial peptide TENGU. Plant Physiol. 2013;162:2005–14. https://doi.org/10.1104/pp.113.218586.

Tsirigotaki A, De Geyter J, Šoštaric N, Economou A, Karamanou S. Protein export through the bacterial Sec pathway. Nat Rev Microbiol. 2017;15:21–36. https://doi.org/10.1038/nrmicro.2016.161.
Vanacker H, Carver TL, Foyer CH. Early H_2O_2 accumulation in mesophyll cells leads to induction of glutathione during the hyper-sensitive response in the barley-powdery mildew interaction. Plant Physiol. 2000;123:1289–300. https://doi.org/10.1104/pp.123.4.1289.
Wang J, Song L, Jiao Q, Yang S, Gao R, Lu X, et al. Comparative genome analysis of jujube witches'-broom Phytoplasma, an obligate pathogen that causes jujube witches'-broom disease. BMC Genomics. 2018a;19:689. https://doi.org/10.1186/s12864-018-5075-1.
Wang N, Pierson EA, Setubal JC, Xu J, Levy JG, et al. The *Candidatus* Liberibacter-host interface: insights into pathogenesis mechanisms and disease control. Ann Rev Phytopathol. 2017;55:451–82. https://doi.org/10.1146/annurev-phyto-080516-035513.
Wang N, Yang H, Yin Z, Liu W, Sun L, Wu Y. Phytoplasma effector SWP1 induces witches' broom symptom by destabilizing the TCP transcription factor BRANCHED1. Mol Plant Pathol. 2018b;19:2623–34. https://doi.org/10.1111/mpp.12733.
Wang Q, Han C, Ferreira AO, Yu X, Ye W, Tripathy S, et al. Transcriptional programming and functional interactions within the *Phytophthora sojae* RXLR effffector repertoire. Plant Cell. 2011;23:2064–86. https://doi.org/10.1105/tpc.111.086082.
Wei HL, Zhang W, Collmer A. Modular study of the type III effector repertoire in *Pseudomonas syringae* pv. tomato DC3000 reveals a matrix of effector interplay in pathogenesis. Cell Rep. 2018;23:1630–8. https://doi.org/10.1016/j.celrep.2018.04.037.
Weisburg WG, Tully JG, Rose DL, Petzel JP, Oyaizu H, Yang D. A phylogenetic analysis of the mycoplasmas: basis for their classification. J Bacteriol. 1989;171:6455–67. https://doi.org/10.1128/jb.171.12.6455-6467.1989.
Yang L, McLellan H, Naqvi S, He Q, Boevink PC, Armstrong M, et al. Potato NPH3/RPT2-Like protein StNRL1, targeted by a *Phytophthora infestans* RXLR effector, is a susceptibility factor. Plant Physiol. 2016;171:645–57. https://doi.org/10.1104/pp.16.00178.
Zhou J, Ma F, Yao Y, Deng M, Chen M, Zhang S, et al. Jujube witches' broom phytoplasma effectors SJP1 and SJP2 induce lateral bud outgrowth by repressing the ZjBRC1-controlled auxin efflux channel. Plant Cell Environ. 2021;44:3257–72. https://doi.org/10.1111/pce.14141.
Zvereva AS, Golyaev V, Turco S, Gubaeva EG, Rajeswaran R, Schepetilnikov MV, et al. Viral protein suppresses oxidative burst and salicylic acid-dependent autophagy and facilitates bacterial growth on virus-infected plants. New Phytol. 2016;211:1020–34. https://doi.org/10.1111/nph.13967.

Ready to submit your research? Choose BMC and benefit from:

- fast, convenient online submission
- thorough peer review by experienced researchers in your field
- rapid publication on acceptance
- support for research data, including large and complex data types
- gold Open Access which fosters wider collaboration and increased citations
- maximum visibility for your research: over 100M website views per year

At BMC, research is always in progress.

Learn more biomedcentral.com/submissions