Lso-HPE1, an Effector of ‘*Candidatus Liberibacter solanacearum*’, Can Repress Plant Immune Response

Julien G. Levy,^{1,†} Rachel Gross,⁴ Azucena Mendoza-Herrera,² Xiaotian Tang,² Kevin Babilonia,³ Libo Shan,³ Joseph C. Kuhl,⁴ Margaret S. Dibble,⁴ Fangming Xiao,⁴ and Cecilia Tamborindeguy^{2,†}

¹ Department of Horticultural Sciences, Texas A&M University, College Station, TX 77843

² Department of Entomology, Texas A&M University, College Station, TX 77843

³ Institute for Plant Genomics and Biotechnology, Department of Plant Pathology and Microbiology, Texas A&M University, College Station, TX 77843

⁴ Department of Plant Sciences, University of Idaho, Moscow, ID 83844

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ABSTRACT

‘*Candidatus Liberibacter solanacearum*’ is a plant pathogen affecting the families Solanaceae and Apiaceae in different parts of the world. ‘*Ca. L. solanacearum*’ is a Gram-negative, fastidious α -proteobacterium that is vectored by different psyllid species. Plant-pathogenic bacteria are known for interfering with the host physiology or defense mechanisms, often by secreting bacterial effectors. Effector proteins are critical for virulence; therefore, the identification of effectors could help with disease management. In this study, we characterized the Sec-translocon-dependent ‘*Ca. L. solanacearum*’-hypothetical protein effector 1 (Lso-HPE1). We compared this protein sequence in the different ‘*Ca. L. solanacearum*’ haplotypes. We predicted the signal peptide and validated its function using *Escherichia coli*’s alkaline phosphatase fusion assay.

Agrobacterium tumefaciens-mediated transient expression in *Nicotiana benthamiana* demonstrated that Lso-HPE1 from ‘*Ca. L. solanacearum*’ haplotypes A and B were able to inhibit the induction of cell death in plants. We also compared gene expression of the Lso-HPE1- transcripts in ‘*Ca. L. solanacearum*’ haplotypes A and B in tomato and in the vector *Bactericera cockerelli*. This work validates the identification of a Sec-translocon-dependent ‘*Ca. L. solanacearum*’ protein possibly involved in suppression of plant cell death.

Keywords: analytical and theoretical plant pathology, *Bactericera cockerelli*, bacteriology, ‘*Candidatus Liberibacter solanacearum*’, Lso haplotype, potato, psyllid, tomato, zebra chips

‘*Candidatus Liberibacter solanacearum*’ is a fastidious Gram-negative bacterium that infects different plant families worldwide. Several ‘*Ca. L. solanacearum*’ haplotypes have been identified infecting solanaceous or apiaceous crops (Glynn et al. 2012; Lin et al. 2012; Nelson et al. 2011). The ‘*Ca. L. solanacearum*’ A and B haplotypes (LsoA and LsoB) are transmitted by the tomato psyllid *Bactericera cockerelli* (Hemiptera: Trioziidae), which is also known as the potato psyllid. These haplotypes infect solanaceous plants in North and Central America and New Zealand (Liefing et al. 2008, 2009), causing damage and increasing costs to potato and tomato production. Last year, the existence of LsoF-infected potato was reported in the United States; however, it has not been determined yet whether this is a psyllid-borne pathogen (Swisher Grimm and Garczynski 2019). In contrast, LsoC, LsoD, and LsoE are transmitted by carrot psyllids and infect apiaceous crops in the Europe and the Mediterranean Basin (Alfaro-Fernández et al. 2012; Tahzima et al. 2014; Teresani et al. 2014). Presently, no commercially acceptable host plant resistance has been identified against any *Liberibacter* pathogen. Therefore, the main strategy to

control these pathogens relies on calendar applications of pesticides. This strategy imposes an additional cost to the industries affected by *Liberibacter* pathogens. Indeed, for example, the cost associated with psyllid control in the Pacific Northwest region of the United States amounts to \$11 million per year (Greenway and Rondon 2018). Hence, novel approaches to control these pathogens are urgently needed.

Although ‘*Ca. L. solanacearum*’ can complete its life cycle in both eukaryotic hosts (the plant and the vector), it is presently unculturable, which makes performing ‘*Ca. L. solanacearum*’ studies difficult. However, the genomes of several ‘*Ca. L. solanacearum*’ haplotypes are available in NCBI (<https://www.ncbi.nlm.nih.gov/genome/genomes/2244>). Those include three LsoA, two LsoB, one LsoC, and one LsoD strain sequences which can help in identifying the molecular basis of ‘*Ca. L. solanacearum*’-host interactions. Nevertheless the genetic determinants that control the host range of different ‘*Ca. L. solanacearum*’ haplotypes in both plants and insect hosts remain unknown.

Differences between ‘*Ca. L. solanacearum*’ haplotypes have been reported in the interaction with plants and vectors (Tamborindeguy et al. 2017). In potato, infection with LsoA or LsoB causes zebra chip (Crosslin et al. 2010; Secor and Rivera-Varas 2004). This disease results in diverse aerial symptoms common to other pathologies. Additionally, tubers from infected plants show a characteristic striped pattern that intensifies after frying (Munyanza et al. 2007). Both LsoA and LsoB haplotypes cause similar symptoms, albeit with differences, and, in both cases, the infection of potato plants results in early plant death (Harrison et al. 2019; Swisher Grimm et al. 2018). An incidental and small sample of LsoC-infected potato that did not show any symptoms was reported in Finland (Haapalainen et al. 2018). In tomato, ‘*Ca. L. solanacearum*’ infection induces spiky apical leaves, chlorosis and

[†]Corresponding authors: J. Levy: julienlevy@tamu.edu; and C. Tamborindeguy ctamborindeguy@tamu.edu

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mottling of leaves, plant stunting, and fruit deformation (Liefting et al. 2009; Rojas-Martinez et al. 2016). Thus, LsoA and LsoB haplotypes represent a valuable system to decipher the molecular basis of ‘*Ca. L. solanacearum*’ pathogenicity because they are transmitted by the same vector and infect the same plant species but present differences in virulence. For instance, LsoB infection causes early death of tomato plants approximately 8 weeks after infection whereas LsoA-infected tomato plants continue to live and can produce fruit as long as they are properly maintained (Mendoza-Herrera et al. 2018).

Bacterial effectors play an important role in promoting pathogenicity during the interaction of diverse plant–pathogen systems (Macho and Zipfel 2015). Bacterial effectors are proteins secreted by pathogens and delivered into the cells of their hosts. These proteins often interfere with the host physiological or immune responses for the benefit of the pathogen to invade or survive in the host (Toruño et al. 2016). Microorganisms that depend on other hosts to complete their life cycle manipulate their hosts to permit colonization. For example, these microorganisms can release effectors to modulate the physiological response of their eukaryotic host; this could be through direct or indirect suppression of the host defense response. To counteract infections, likely as a consequence of evolution, some plant species further evolve specific immune receptors to recognize these effectors to trigger immune responses. In this case, the effectors function as avirulence factors and trigger effector-triggered immunity (ETI) upon recognition by plant resistance (R) proteins (Jones and Dangl 2006). One major group of R proteins are the nucleotide-binding site leucine-rich repeat (NBS-LRR) type, which are similar to the family of mammalian nucleotide-binding oligomerization domain-LRR proteins that function in immune responses as well (DeYoung and Innes 2006). In plants, R proteins can trigger programmed cell death as a defense response, also known as hypersensitive response (HR), upon recognition of pathogen avirulence effectors. Although the mechanism of R protein activation is not completely understood, it is believed that the R protein undergoes a conformational change to switch from an inactive state to an active state which can then lead to programmed cell death to prevent the pathogen from spreading in the host (Du et al. 2012). Effector discovery and later characterization of the effector functions and host targets are pivotal to the development of disease resistance strategies.

‘*Ca. L. solanacearum*’ is related closely to ‘*Ca. Liberibacter*’ spp. responsible for huanglongbing, also known as citrus greening disease. These include ‘*Ca. L. asiaticus*’, ‘*Ca. L. africanus*’, and ‘*Ca. L. americanus*’. Whereas, in ‘*Ca. L. asiaticus*’, several effectors have been identified and tested for different functions (Clark et al. 2018; Jain et al. 2018; Pitino et al. 2016), only ‘*Ca. L. solanacearum*’ serrallysin has been reported as a potential effector (Ravindran et al. 2018).

In this study, we focused on a ‘*Ca. L. solanacearum*’ gene encoding a hypothetical protein that was identified as a putative secreted protein based on bioinformatic analyses (Prasad et al. 2016). First, we compared the amino acid sequence of the encoded protein among the different ‘*Ca. L. solanacearum*’ haplotypes and *Liberibacter* genomes. Second, we evaluated the expression of the transcripts in LsoA- and LsoB-infected tomato plants and psyllids. Third, we tested whether the ‘*Ca. L. solanacearum*’ protein encoded a functional secretion signal peptide (SP). Finally, we evaluated whether the protein could act as an effector that interferes with plant defenses. To do this, we tested the ability of the protein to suppress plant defense mechanisms such as Prf^{D1416V}- or Bax-induced cell death response in *Nicotiana benthamiana* using a transient expression system. In tomato, the NBS-LRR resistance protein Prf can induce cell death upon recognition of pathogen avirulence effectors (Salmeron et al. 1996). A Prf variant with a single amino acid substitution, Prf^{D1416V}, mimics the effector-induced active state of the protein and causes bacteria- or effector-independent programmed cell death when transiently expressed in leaves of

N. benthamiana (Du et al. 2012). This system has been developed as a tool to test effectors. Similarly, Bax is a mammalian protein that functions as a regulator of apoptosis and can cause cell death and accumulation of the defense-related protein PR1 when expressed in *N. benthamiana* (Lacomme and Santa Cruz 1999). This system can also be used to evaluate the function of putative effectors (Xiang et al. 2016).

MATERIALS AND METHODS

Plant material. Tomato (*Solanum lycopersicum*) (Money-maker) and *N. benthamiana* seed were grown in Sunshine Mix (Sun Gro Horticulture Canada) on light shelves under a cycle of 16 h of light and 8 h of darkness at 21 to 24°C.

‘*Ca. L. solanacearum*’-hypothetical protein effector 1 sequence validation. *B. cockerelli* colonies harboring LsoA or LsoB (Yao et al. 2016) were maintained on tomato plants as was previously described (Ibanez et al. 2017). DNA from pools of 10 insects from each colony was purified using the cetyltrimethylammonium bromide method (Nachappa et al. 2011).

The ‘*Ca. L. solanacearum*’-hypothetical protein effector 1 (Lso-HPE1) gene sequence (ADR52633.1, formerly known as CKC_04420) was amplified by PCR using DNA from each colony. Briefly, 50 ng of DNA were combined with 0.2 µl of Phusion High-Fidelity DNA Polymerase (New England Biolabs, Ipswich, MA), 4 µl of 5× Phusion HF Buffer, 0.4 µl of 10 mM dNTPs, 1 µl of each primer at 10 nM (Table 1), and molecular-grade water. The PCR conditions were 98°C for 30 s; followed by 35 cycles of 98°C for 10 s, 55°C for 30 s, and 72°C for 30 s; and a final extension at 72°C for 6 min. The amplicons were purified using a PureLink PCR Purification Kit (Invitrogen, Carlsbad, CA) and ligated into a pGEM-T easy vector using the pGEM-T Easy cloning kit (Promega Corp., Madison, WI). The ligation was transformed into One Shot TOP10 competent cells (Invitrogen). Transformed colonies were selected and plasmid DNA extractions from at least three colonies were performed using a PureLink Quick Plasmid Miniprep Kit (Invitrogen) and sequenced by Eton Bioscience Inc. (San Diego, CA). The obtained sequences were compared with the ‘*Ca. L. solanacearum*’-sequenced genomes.

Protein sequence analysis. Bioinformatic analysis of the Lso-HPE1 protein sequence to predict SPs was performed using SignalP version 5.0 (Almagro Armenteros et al. 2019) with default settings for Gram-negative bacteria. A Blastp search analysis was performed to identify the most similar proteins in other *Liberibacter* spp.

Phylogenetic analysis. For the phylogenetic analysis, sequences were aligned with ClustalW using the MEGA 5.2 software (Tamura et al. 2011). The phylogenetic reconstruction was done by Bayesian inference using MrBayes 3.2 (Ronquist et al. 2012). Markov-chain Monte Carlo runs were carried out for 1,000,000 generations, and the first 25% of sampled trees were discarded as burn-in. Tree information was visualized and edited using FigTree v1.4.3 (<http://tree.bio.ed.ac.uk/software/figtree>). The ‘*Ca. L. asiaticus*’ protein identified by Blast analysis (WP_012778427) was used as outgroup.

Alkaline phosphatase assays. The 78 nucleotide sequence coding for the putative SP plus 15 additional nucleotides encoding 5 extra amino acids of the Lso-HPE1 protein was amplified by PCR. The PCR product was purified on a column using the QIAquick kit (Qiagen, Valencia, CA), digested with the *SacI* and *MfeI* restriction enzymes (NEB), and cloned into a previously digested pJDT-SDM1 vector using T4 DNA ligase (NEB) to obtain an in-frame gene fusion with the gene *phoA* in the vector. One Shot TOP10 competent cells were transformed with the resulting construct. The transformed bacteria were selected on Lysogeny broth (LB) agar plates containing ampicillin at 100 µg/ml. The insert was verified by PCR and sequencing. The transformed bacteria were tested for alkaline phosphatase (PhoA) activity as previously published by

Ammerman et al. (2008) as follows. Bacteria were grown on LB agar plates containing ampicillin at 100 µg/ml, 5-bromo-4-chloro-3-indolyl phosphate (5-BCIP) at 90 µg/ml as chromogenic substrate, and 75 mM Na₂HPO₄ to block endogenous phosphatase activity. *Escherichia coli* bacteria transformed with the empty plasmid pJTD-SDM1 were used as negative control. We also verified the secretion using the overnight liquid culture of Lso-HPE1-phoA and the pJTD1 empty vector. For that, 1.5 ml of liquid culture was centrifuged for 15 min at 3,000 rpm. The supernatants were recovered, filtered through a 0.2-µm filter (VWR), and incubated with 5-BCIP at 90 µg/ml at room temperature for 2 h (Supplementary Fig. S1). We also spotted the filtered supernatants on LB-ampicillin plates and verified that no bacterium grew after an overnight incubation at 37°C, showing that the supernatants were free of bacterial contamination.

Cloning of Lso-HPE1 into pEarlyGate 101 and *Agrobacterium* transformation. Lso-HPE1 from psyllids harboring LsoA was amplified as previously described using the Phusion High-Fidelity DNA Polymerase. Lso-HPE1 from psyllids harboring LsoB was amplified using Platinum Taq Polymerase (Thermo Fisher Scientific, Waltham, MA). Primers used are described in Table 1. The amplicons were cloned in the pENTR vector by TOPO cloning according to manufacturer's directions (Thermo Fisher Scientific) and verified by sequencing. The purified pENTR vector containing Lso-HPE1 was digested with *Mlu*I, and the insert was then transferred to pEarlyGate 101 (pEG101) with the GateWay LR Clonase II enzyme (Thermo Fisher Scientific) following the manufacturer's recommendations and verified by sequencing. The *Agrobacterium tumefaciens* strain LBA4404 was transformed following the heat shock method.

A. *tumefaciens*-mediated transient expression. The *Agrobacterium*-mediated transient expression assays were performed as follows. All bacteria were grown for 24 h at 28°C and 280 rpm in 3 ml of LB supplemented with kanamycin (50 µg/ml), 10 mM morpholineethanesulfonic acid (MES), and 20 µM acetosyringone. Cells were pelleted and resuspended in fresh buffer containing 10 mM MES, 200 µM acetosyringone, and 10 mM MgCl₂. Cells were resuspended at an optical density at 600 nm (OD₆₀₀) = 0.7, except those containing the PrF^{D1416V}, which were resuspended at OD₆₀₀ = 0.3. *Agrobacterium* suspensions were infiltrated into 5-week-old *N. benthamiana* leaves using 2-ml disposable syringes. *Agrobacterium* suspensions were either infiltrated alone or in coinfiltration. Western blot analyses were conducted to verify Lso-HPE1 protein expression (Supplementary Figs. S1, S2, and S3). After infiltration, plants were maintained on light shelves at room temperature (21 to 24°C). We repeated each experiment three times and included six to eight plants per experiment. We infiltrated six to eight leaves per plant. Cell death was scored at variable time points following infiltration, usually at 4, 6, and 8 days postinfiltration. To evaluate the ability of Lso-HPE1 to disrupt cell death, coinfiltrations were performed with pCXSN-Bax and PrF^{D1416V} separately. As a control, pCXSN-Bax and PrF^{D1416V} were coinfiltrated with pEG101 empty vector (negative control) and pBETX-AvrPto,

separately. The cell death response was scored on a scale of 0 (no reaction) to 1 (cell death); a score of 0.5 was used if partial cell death reaction occurred; that is, when spots of cell death developed in the infiltrated area (Supplementary Fig. S2). The average of cell death per treatment was calculated and is reported.

Gene expression analysis. *B. cockerelli* colonies carrying either LsoA or LsoB were maintained and tested in our laboratory as previously described (Nachappa et al. 2014; Yao et al. 2016). Ten '*Ca. L. solanacearum*'-infected insects from each colony were pooled. Insect RNA samples were extracted and processed as previously published (Ibanez et al. 2014). Total RNA from each pool of insects was purified using RNeasy Mini kit (Qiagen) followed by DNase treatment with RNase-Free DNase (Qiagen). Three pools per colony were analyzed.

LsoA- and LsoB-infected plant samples were collected from plants previously infested with LsoA- or LsoB-infected insects. Leaf tissue was collected from the top-most fully expanded leaf of each plant 3 weeks postinfection and immediately flash frozen in liquid nitrogen. After the leaf sample was collected, each plant was kept until the end of the experiment in order to collect plant tissue for DNA extraction to verify '*Ca. L. solanacearum*' presence or absence. '*Ca. L. solanacearum*' in plants can be detected 4 weeks after *B. cockerelli* infestation (Levy et al. 2011). The flash-frozen tissue samples were ground to fine powder. Total RNA extraction was performed using the RNeasy Plant Mini Kit (Qiagen) according to the manufacturer's instructions. The RNA samples were treated with RNase-Free DNase (Qiagen).

To quantify gene expression in plants and insects, quantitative PCR (qPCR) was performed following previously published methods (Ibanez et al. 2014). Total RNA (50 ng) was reverse transcribed into cDNA and the qPCR was performed in the same reaction using the Luna Universal one-step RT-qPCR kit (NEB), following the manufacturer's protocol. The amplification protocol consisted of one step at 55°C for 10 min (reverse transcription), one step at 95°C (initial denaturation), and 40 cycles of 95°C for 15 s and 60°C for 30 s using a QuantStudio 6 Flex Real-Time PCR System (Thermo Fisher Scientific). Each reaction contained 100 ng of RNA, 0.25 µM each primer (Table 1), 5 µl of Luna Universal One-Step Reaction Mix (2×), 0.5 µl of Luna WarmStart RT Enzyme Mix (20×), and molecular-grade water. Reactions for all samples were performed in duplicate, with negative controls in each run. The mean threshold cycle (Ct) values and standard deviation were calculated. The Rec A (ADR52764.1, CKC_05085) primers were used as bacterial housekeeping gene control (Ibanez et al. 2014). ΔCt was calculated for the average of three biological replicates for each condition. Mean and standard error of the mean were calculated and reported. Quantitative PCR analyses were analyzed using one-way analysis of variance (ANOVA) with Tukey's posthoc test in R. Levene's test was carried out to test for equality of variances and the assumption was met.

The accession numbers for the different sequences are as follows: LsoA = WP_045960716, KJZ81751.1(NZ1), and KQC48998 (RSTM); LsoC = WP_076969720, 112aa FIN111, ONI58619.1, and

TABLE 1. Primers used in the study

Primer name	Sequence 5'-3'	Comments
Lso-HPE1F (<i>Sac</i> I)	CACGAGCTCATGAATTTTAAATGTTAGC	Cloning in PJDT1
Lso-HPE1R (<i>Mfe</i> I)	CACCAATTGTATTTTCATAAAGTACTGTG	Cloning in PJDT1
PJDT1-F	CAGGAAACAGCTATGAC	Sequencing of PJDT1
PJDT1-R	CGCTAAGAGAATCACGCAGAGC	Sequencing of PJDT1
Gene Lso-HPE1 F	AATGGCGGGAGAGTATTAAA	Sequencing of Lso-HPE1
Gene Lso-HPE1 R	AATATGAAAAATCCATCGGAT	Sequencing of Lso-HPE1
qPCR-F	TGTCAAATGTCTAAGTTGCGAATTG	Expression of Lso-HPE1
qPCR-R	ATCGGTCATGACTATGGCATT	Expression of Lso-HPE1
Gateway Lso-HPE1F (LsoA and LsoB)	CACCATGAATTTTAAATGTTAGC	Gateway cloning Lso-HPE1
Gateway Lso-HPE1R (LsoA)	GTGACGCGCAGAATCATTA	Gateway cloning Lso-HPE1LsoA
Gateway Lso-HPE1F (LsoB)	CTGATGCGCAGAATCCTTA	Gateway cloning Lso-HPE1LsoB

Therefore, to validate the presence of an active SP in Lso-HPE1, we tested the motif in *E. coli* using the *phoA* gene fusion assay. Although the control *E. coli* cells with an empty pJDT1-SDM vector remained white, the bacteria with the fusion Lso-HPE1 SP-*phoA* turned blue after a 6-h incubation (Fig. 3). We confirmed the function of the SP by testing the phosphatase activity using a filtered supernatant from a bacterial liquid culture (Supplementary Figure S1). This validated the activity of the Lso-HPE1 SP.

Fig. 2. Phylogenetic analysis of *Liberibacter* homologs to ‘*Candidatus* *Liberibacter solanacearum*’ (Lso)–hypothetical protein effector 1 protein under Bayesian inference. Scale bar indicates branch length. The ‘*Ca. L. asiaticus*’ (CLas) protein was used as outgroup. Numbers at the nodes denote posterior probabilities (support values > 0.7 are shown).

Fig. 1. Alignment of the homologs of ‘*Candidatus* Liberibacter solanacearum’ (Lso)–hypothetical protein effector 1 identified by Blast analysis in Liberibacter genomes. Proteins identified in ‘*Ca. L. solanacearum*’ genomes were highly similar. ‘*Ca. L. asiaticus*’ (CLAs) protein was smaller. Row cons.LsoA/B is the consensus sequence between LsoA and LsoB, in which dots (.) label positions with similar residues and asterisks (*) label positions in the alignment with identical residues. The arrow between amino acids 20 and 21(GKA-QS) marks the putative cleavage site of the signal peptide.

GAGCTC ATGAATTTTAAATGTTAGCAGTAATATTATTGCGTTCAGCGCTCAAGTAGGAAAAGCACAGTCAGTTTATGAAATA CAATTG GCT...

E L M N F L M L A V I L F A F S A Q V G K A Q S V Y E I Q L A

Fig. 3. Alkaline phosphatase (PhoA) assay validated the prediction of the signal peptide of '*Candidatus Liberibacter solanacearum*'-hypothetical protein effector 1 (Lso-HPE1). The top panel shows the results of the assay: four independent *Escherichia coli* clones with the fusion Lso-HPE1-phoA changed color while two independent clones without the signal peptide (SP) remained white when grown on Lysogeny broth containing 5-bromo-4-chloro-3-indolyl phosphate. The bottom panel is a diagram of the fusion of the Lso-HPE1 SP and the *phoA* gene. The vertical line in the amino acid sequence represents the predicted cleavage site in the Lso-HPE1 protein. Underlined in the nucleic acid and the protein sequences are the restriction sites introduced for the cloning.

Fig. 4. Cell death induction after *Agrobacterium* infiltration. Pictures were taken 7 days postinfiltration. The picture shows the cell death phenotype 7 days after infiltration. Each column is labeled with infiltration construct, in the following order: **A**, PrfD1416V alone (Prf) showed cell death (score 1), coinfiltration of PrfD1416V–Peg101 empty vector showed cell death (score 1), coinfiltration of PrfD1416V and '*Candidatus Liberibacter solanacearum*'-hypothetical protein effector 1 (Lso-HPE1)-A did not show cell death (score 0), and coinfiltration of PrfD1416V–Lso-HPE1-B did not show cell death (score 0). **B**, Bax alone showed cell death (score 1), coinfiltration of Bax–Lso-HPE1-A and coinfiltration of Bax–Lso-HPE1-B did not show cell death (score 0). **C**, Infiltration of Lso-HPE1-A and Lso-HPE1-B alone did not show cell death (score 0).

Prf^{D1416V} and Lso-HPE1 from LsoA or from LsoB resulted in a reduction of cell death development. Although the average cell death score was 0.92 when Prf^{D1416V} was infiltrated alone, coinfiltration of Prf^{D1416V} with Lso-HPE1 from LsoA or with Lso-HPE1 from LsoB scored, on average, 0.36 and 0.26, respectively (Fig. 4A). As a positive control, Prf^{D1416V} was coinfiltrated with AvrPto and scored, on average, 0.15 in the cell death assays, whereas coinfiltration of pEG101 empty vector with Prf^{D1416V} scored, on average, 0.85.

Similarly, coinfiltration of Bax and Lso-HPE1 from LsoA and LsoB resulted in reduction of cell death. In this case, the average cell death score was 0.8 for Bax alone whereas the coinfiltrations of Bax with Lso-HPE1 from LsoA or Lso-HPE1 from LsoB scored, on average, 0.33 and 0.36, respectively (Fig. 4B).

The infiltration of Lso-HPE1 from LsoA or from LsoB alone (Fig. 4C) did not induce cell death.

Gene expression. Lso-HPE1 expression was compared between LsoA- and LsoB-infected plants and between LsoA- and LsoB-infected psyllids. The gene was expressed in both plants and insects infected with LsoA or LsoB. The highest relative expression was measured in LsoB-infected insects (Fig. 5). A two-way ANOVA was carried out on the relative expression of Lso-HPE1 by host type and '*Ca. L. solanacearum*' haplotype. There was a statistically significant interaction between the effects of the host type and the haplotype ($F [1, 8] = 24.66, P = 0.001$). Tukey's honestly significant difference posthoc tests determined that Lso-HPE1 relative expression was significantly higher in LsoB-infected psyllids compared with LsoA-infected psyllids ($P = 0.009$) and LsoB-infected tomato ($P = 0.006$).

Fig. 5. Relative expression of '*Candidatus Liberibacter solanacearum*' (Lso)-hypothetical protein effector 1 in LsoA- and LsoB-infected tomato plants and psyllids. Gene expression was quantified using reverse-transcription quantitative PCR and analyzed using the Δ threshold cycle (ΔCt) method. mRNA expression level was normalized relative to '*Ca. L. solanacearum*' RecA. Bars represent mean $\Delta Ct \pm$ standard error of the mean ($n = 3$). Letters indicate significant differences in gene expression as determined using two-way analysis of variance and Tukey's posthoc analyses.

DISCUSSION

Despite the economic importance of diseases caused by '*Ca. L. solanacearum*' and the expanding geographical range of these pathogens, very little is known about the molecular basis of their pathogenicity. Bacterial effectors able to suppress plant immune responses have been characterized (Jamir et al. 2004). Furthermore, Sec-translocon-dependent bacterial effectors were also shown to play a key role in disease development during infection with intracellular pathogens such as phytoplasmas (Hogenhout et al. 2009). Similarly to *Liberibacter* spp., phytoplasmas are also vector-borne, phloem-restricted, plant-pathogenic bacteria characterized by a reduced genome. Therefore, we hypothesize that *Liberibacter* spp. which encode a complete Sec secretion system (Ibanez et al. 2014) might also encode Sec-translocon-dependent proteins that play key roles in plant infection. Here, we have identified and characterized a Sec-translocon-dependent secreted '*Ca. L. solanacearum*' protein which may play a role in suppressing protein-triggered cell death, part of the plant disease resistance response.

Findings from this study suggest that this protein is conserved among the '*Ca. L. solanacearum*' pathogens but not present in the other *Liberibacter* spp. The absence of Lso-HPE1 in two of the available LsoA genomes is probably due to the lower genome coverage of these assemblies. Indeed, the protein was found in the LsoB strain ZC1, the LsoA strains NZ1 and RSTM, as well as in the LsoC and LsoD genomes, in which it shows a high degree of similarity. Remarkably, a 23-amino-acid-long motif in the C-terminal region of the protein presents higher conservation between LsoA and LsoB than among other Lso-HPE1 proteins. We could speculate that this motif might be of interest for the protein activity, and could be associated with host or vector specificity. Indeed, these differences correlate with the geographic location and host association of these pathogens: '*Ca. L. solanacearum*' haplotypes C and D are associated with apiaceous crops in Europe, while haplotypes A and B are associated with solanaceous crops in North America (Haapalainen 2014). With only 43.58% identity, the most similar '*Ca. L. asiaticus*' protein is probably not a homolog protein. However, this '*Ca. L. asiaticus*' protein, which lacks the C-terminal domain that differentiates Lso-HPE1 proteins, is predicted to be secreted by the Sec-translocon system (Prasad et al. 2016). The cellular localization of this protein was shown to be temperature dependent in *Agrobacterium*-mediated transient expression assays in *N. benthamiana* epidermal cells (Liu et al. 2019).

SignalP predicted a 20-amino-acid-long cleaved SP but this SP did not have the structure of other SEC targeting signals (Cranford-Smith and Huber 2018). However, proteins lacking this type of SP can be recognized by the Sec machinery. Therefore, it was important to validate whether the predicted SP was active. The bioassay performed with the 25 amino acids of the Lso-HPE1 protein N terminus demonstrated a very strong secretion when compared with other secretion screening studies; in particular, the ones performed with '*Ca. L. asiaticus*' proteins (Prasad et al. 2016).

To examine the role of Lso-HPE1 in plant immunity, the proteins encoded by LsoA and LsoB were transiently expressed in *N. benthamiana* leaves. Neither of the Lso-HPE1 proteins triggered observable cell death when expressed alone, while they both were able to suppress cell death induced by two elicitors Bax and Prf^{D1416V} in coinfiltration assays. These results led us to hypothesize that Lso-HPE1 can play a role in inhibiting immune responses similarly to AvrPto, a well-characterized bacterial effector involved in disrupting plant responses. In the present experiments, coinfiltration of Prf^{D1416V} and Lso-HPE1 resulted in fewer cases of cell death compared with the infiltration of Prf^{D1416V} alone or coinfiltration with the empty vector. Because Lso-HPE1 was able to disrupt the HR induced by Prf^{D1416V}, an autoactive variant of the Prf NBS-LRR resistance protein, this bacterial protein appears to interfere with ETI; thus, Lso-HPE1 could act in the signaling pathway downstream of the activation of R receptors or during

the development of the cell death phenotype. The mechanisms involved in Bax-induced cell death in plants remain unknown. Bax is a mammal proapoptotic protein that is able to induce cell death in plants and yeast (Lacomme and Santa Cruz 1999; Zha et al. 1996). However, because Lso-HPE1 was able to suppress Bax-induced cell death, we speculate that this protein might act as a general cell death suppressor. The use of these two cell death inducers can help in deciphering the molecular role that bacterial effectors play in plants. For instance, potato cyst nematode effector RHA1B is able to suppress Prf^{D1416V} but not Bax-induced cell death (Kud et al. 2019), whereas the well-characterized *Pseudomonas* effector AvrPto is able to suppress the cell death induced by both proteins.

Bax induces cell death in a wide range of organisms; therefore, the ability of Lso-HPE1 to suppress Bax-induced cell death could imply that this bacterial protein is also able to suppress cell death in its vector, the potato psyllid. Indeed, programmed cell death is a common defense mechanism in insects (Hillyer 2016). However, apoptosis is not induced in the midgut of potato psyllids by '*Ca. L. solanacearum*' (Tang and Tamborindeguy 2019). This lack of apoptosis could result from the activity of Lso-HPE1 or other '*Ca. L. solanacearum*' effectors. Thus, it appears that testing the ability of putative bacterial effectors to suppress Bax-induced cell death in *N. benthamiana* could be used as a first screening method to identify candidate proteins involved in the interaction between plant pathogens and their vectors. Vectors of plant pathogens are usually hemipterans, for which genetic tools are sorely lacking.

Finally, because the expression of bacterial effectors are usually expressed during infection, we evaluated the expression of the Lso-HPE1 in the vector and in tomato. We detected a higher expression of Lso-HPE1 in LsoB-infected insects compared with LsoA-infected insects and LsoB-infected plants. However, we found a higher but not significant relative expression of Lso-HPE1 in LsoA-infected tomato plants than LsoB-infected plants. The expression of this gene in insects supports the hypothesis that its role might include modulating the vector immune response. It is possible that Lso-HPE1 has a differential active role in insects and in plants. Further experiments are needed to identify the host proteins that interact with Lso-HPE1.

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