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SHORT COMMUNICATION

Expression of putative effectors of different *Xylella fastidiosa* strains triggers cell death-like responses in various *Nicotiana* model plants

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Abstract

The wide host range of *Xylella fastidiosa* (Xf) indicates the existence of yet uncharacterized virulence mechanisms that help pathogens to overcome host defences. Various bioinformatics tools combined with prediction of the functions of putative virulence proteins are valuable approaches to study microbial pathogenicity. We collected a number of putative effectors from three Xf strains belonging to different subspecies: Temecula-1 (subsp. *fastidiosa*), CoDiRO (subsp. *pauca*), and Ann-1 (subsp. *sandyi*). We designed an in planta *Agrobacterium*-based expression system that drives the expressed proteins to the cell apoplast, in order to investigate their ability to activate defence in *Nicotiana* model plants. Multiple Xf proteins differentially elicited cell death-like phenotypes in different *Nicotiana* species. These proteins are members of different enzymatic groups: (a) hydrolases/hydrolase inhibitors, (b) serine proteases, and (c) metal transferases. We also classified the Xf proteins according to their sequential and structural similarities via the I-TASSER online tool. Interestingly, we identified similar proteins that were able to differentially elicit cell death in different cultivars of the same species. Our findings provide a basis for further studies on the mechanisms that underlie both defence activation in Xf resistant hosts and pathogen adaptation in susceptible hosts.

KEYWORDS

cell death, effectors, innate immunity, PTI, resistance, *Xylella*

Plants respond to invading pathogens by exploiting their innate immune system. Microbe-associated molecular pattern (MAMP)-triggered immunity (MTI) and effector-triggered immunity (ETI) have been described as the two main layers of defence during the infection of a host (Cui et al., 2015; Duxbury et al., 2016; Jones & Dangl, 2006; Mermigka et al., 2020). Recent studies have proposed a revised version of the zig-zag model of plant innate immunity introduced by Jones and Dangl (2006), strongly indicating the existence of a crosstalk between MTI and ETI. ETI potentiates MTI responses and vice versa (Katagiri & Tsuda, 2010; Ngou et al., 2021). MTI is

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responsible for the detection of pathogen-associated molecular patterns (PAMPS)/MAMPs and/or damage-associated molecular patterns (DAMPs) via specific cell surface-localized pattern recognition receptors (PRRs) (Couto & Zipfel, 2016). MTI defensive outcomes include production of reactive oxygen species (ROS), calcium influx, activation of mitogen-activated protein kinases (MAPKs), chromatin remodelling, differential regulation of gene expression, and callose deposition (Lu et al., 2015; Mur et al., 2008; Pardal et al., 2021; Stotz et al., 2014). On the other hand, ETI relies on intracellular receptors called NOD-like receptors (NLRs; Ma et al., 2018; Sarris et al., 2016). NLRs function as an interior surveillance system, monitoring outcomes mediated by pathogen-derived effector molecules and mobilizing or boosting even stronger responses usually related to hypersensitive response (HR) cell death (Jones et al., 2016). Collectively, these responses restrict pathogens to the site of the infection and prevent disease development.

In order to meet their nutritional needs and proliferate effectively inside the host, several pathogens have evolved to secrete virulence factors—known as effectors—directly into the host cell cytoplasm or into the extremely hostile apoplastic space (Mooney et al., 2021). Effector proteins are key aspects of plant–pathogen interactions, potentially controlling the host range through their recognition by the plant surveillance system (Büttner & Bonas, 2010; Reddy et al., 2019). In several cases, interaction of apoplastic pathogen effectors with plant PRRs has been associated with induction of immune responses and programmed cell death (PCD) (van der Burgh & Joosten, 2019). For instance, the apoplastic effectors Chp-7 and ChpG of *Clavibacter michiganensis* elicit PCD when they are secreted to the apoplast, but not when they are expressed in the host cytoplasm (Lu et al., 2015). Similarly, the apoplastic effectors Avr2 and Avr4 from the fungal pathogen *Cladosporium fulvum* are recognized by the receptor-like proteins (RLPs) Cf-2 and Cf-4, respectively, in *Solanum lycopersicum* and trigger a strong defence response, including PCD (Ilyas et al., 2015; Postma et al., 2016; Song et al., 2009). There are additional virulent extracellular effectors related to PCD phenotypes in plants; however, the exact mechanisms of innate immunity underlying their perception remain deeply uncharacterized (Lu et al., 2015; Nissinen et al., 2009).

Xylella fastidiosa (Xf) was first described as the causal agent of Pierce's disease in grapes and it is an extremely dangerous plant-pathogenic bacterium worldwide (Chatterjee et al., 2008; Hopkins & Purcell, 2002; Rapicavoli et al., 2018; Sicard et al., 2018). Xf is a gram-negative, slow-growing, and facultative anaerobic bacterium that has been a subject of interest due to its economic impact (Chatterjee et al., 2008; Shriner & Andersen, 2014). Xf has an extremely extended host range that consists of nearly 600 plant species (Baldi & La Porta, 2017; EFSA, 2018), including *Citrus sinensis*, *Cyanococcus* spp., *Nerium oleander*, *Olea europaea*, and *Vitis vinifera* (EFSA, 2018; Huang et al., 2020; Schneider et al., 2020).

Xf and other extremely important pathogenic bacteria, including *Clavibacter*, *Curtobacterium*, *Erwinia*, *Pantoea*, *Ralstonia*, and close relative *Xanthomonas*, form a group commonly described as vascular wilt pathogens (Agrios, 2005). Xf manages to enter the host's xylem using specific insect vectors, after having successfully colonized their gut (Agrios, 2005; Chatterjee et al., 2008; Roper, 2011). The plant surveillance system remarkably attains recognition and restriction of vascular wilt pathogens in a tissue mostly surrounded by dead cells (Agrios, 2005). This type of immune response primarily relies on xylem parenchyma cells (Yadeta & Thomma, 2013). Living cells surrounding colonized xylem vessels detect pathogens or damage-associated host molecules and trigger downstream immune responses (Agrios, 2005; Yadeta & Thomma, 2013). Successful vascular wilt pathogens achieve spread throughout xylem vessels by degrading cell walls and pit membranes and invading parenchyma cells while resistant hosts detect MAMPs, DAMPs, or secreted virulence factors and prevent systemic spread (Choi & Klessig, 2016). It is noteworthy that an already well-characterized immune receptor (PRR) from the model plant *Arabidopsis thaliana*, known as ELONGATION FACTOR-TU RECEPTOR (EFR), which recognizes the conserved bacterial PAMP EF-Tu and derived elf peptides, was recently shown to confer increased resistance against Xf when expressed in sweet orange, a conditionally susceptible host of this microbe (Mitre et al., 2021).

While there are emerging studies assessing the lifestyle, host specificity, and colonization strategies of this pathogen, less progress has been accomplished in the field of the molecular host–pathogen interactions (Roper et al., 2019). Similarly, the individual roles of putative virulence proteins secreted by Xf in subverting the host's immune machinery and how this leads to disease development is still poorly understood (Chatterjee et al., 2008; Gouran et al., 2016; Nascimento et al., 2016; Rapicavoli et al., 2018; Roper et al., 2019; Zhang et al., 2015).

Xf lacks a type III secretion system (T3SS), a common bacterial machinery for translocation of virulence factors from the pathogen's cytosol directly into the host's intracellular environment. However, Xf possesses type I, II, IV, and V secretion systems (Simpson, 2000; Van Sluys et al., 2003). The Xf 12-protein type II secretion system (T2SS), which is similar to the T2SS of its close relatives of the *Xanthomonas* group, is considered to act as the main source of its pathogenicity (Rapicavoli et al., 2018). Proteases and cell wall-degrading enzymes (CWDEs) are often secreted by the T2SS, while mutations in essential components of the secretion mechanism usually lead to avirulent phenotypes (Rapicavoli et al., 2018).

In this study, using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database, we searched for homologues of various known type II effector genes of pathogenic microorganisms that could be present in several sequenced Xf genomes. This process resulted in the selection of 19 putative Xf type II effectors originating from three different strains belonging to different Xf subspecies for further study (Table S1).

Gene evolution is a process that involves mechanisms such as gene duplications and horizontal gene transfers, which resulted in the hypothesis that sequence-unrelated genes may have high similarity in their tertiary folding and furthermore have the same function in pathogen virulence (Andrie et al., 2008; de Guillen et al., 2015). Based on this hypothesis, the selected Xf proteins were uploaded

to the I-TASSER online server. Of the output, we selected and then compared (a) the proteins with the highest sequence similarity and (b) the proteins with the highest structural similarity based on the template modelling-score (Roy et al., 2009; Yang et al., 2015; Yang & Zhang, 2015) (Figure 1, Figure S1).

To test whether these proteins could elicit PCD after their delivery into the plant apoplast, we first synthesized the corresponding genes including silent mutations where needed to domesticate the sequences making them compatible with the Golden Gate cloning system. Then, we cloned the synthesized genes of interest in an *Agrobacterium*-mediated transient expression system (Figure 2a; Figure S2). The gene expression in this system was under the transcriptional regulation of the constitutive CaMV 35S promoter. To ensure secretion of the protein into the apoplast, we fused the selected Xf proteins to the secretion peptide of tobacco Pathogenesis-Related protein 1a (PR1a) (Lu et al., 2015). The PR1a secretion peptide is cleaved upon secretion to the apoplast (Lu et al., 2015). We generated 19 such constructs to screen for PCD symptoms by the selected Xf proteins. For our screening we used three *Nicotiana tabacum* cultivars (N34'4, Petit Gerard, and Xanthi), *Nicotiana benthamiana*, and six *Nicotiana sylvestris* ecotypes. As a positive control we used the *Clavibacter michiganensis* apoplastic effector Chp7, which has been shown to elicit a PCD response upon its secretion to the apoplast by the PR1a secretion peptide (Lu et al., 2015). Furthermore, the type III effector XopQ of *Xanthomonas campestris* pv. *vesicatoria* served as a secondary positive control, due to its known ability to elicit HR in both *N. tabacum* and *N. benthamiana* (Adlung et al., 2016). *Agrobacterium*-mediated transient expression of the β-glucuronidase (*GUS*) reporter gene in these species did not elicit cell death and it was used as a negative control for our transient expression assays.

Our screening revealed nine proteins that are known or predicted to be type II-secreted by Xf and were able to elicit PCD phenotypes in different *Nicotiana* species (Figure 2b). Most of these proteins successfully elicited PCD 4 days postinoculation (dpi) in all three *N. tabacum* cultivars used and four out of six *N. sylvestris* ecotypes. Interestingly, all nine effectors tested for induction of PCD displayed divergence in distinct *N. sylvestris* ecotypes, while the protein encoded by D934_09300 was able to elicit cell death in *N. tabacum* cultivars N34'4 and Xanthi leaves but not in cv. Petit Gerard, indicating a form of specificity in this response. Moreover, signs of cell death were entirely missing from *N. benthamiana* leaves, suggesting that the responses observed in *N. sylvestris* and *N. tabacum* are most likely not a result of cytotoxic effects. In order to validate this, we produced preliminary reverse transcription-quantitative PCR (RT-qPCR) data indicating the relative expression levels of two defence-related genes, *PR1a* and Plant defensin 1.2 (*PDF1.2*) (Abbas et al., 2020; Rivière et al., 2008), in *N. tabacum* N34'4 leaves transiently expressing four Xf putative effectors (one for each group; Table 1). Our analysis revealed a significant up-regulation of both defence markers 72 h postinoculation (hpi) (Figure S3). Apart from these nine putative effectors, 10 more proteins were studied but did not elicit PCD in any plant species/cultivars tested in this study (Figure S4).

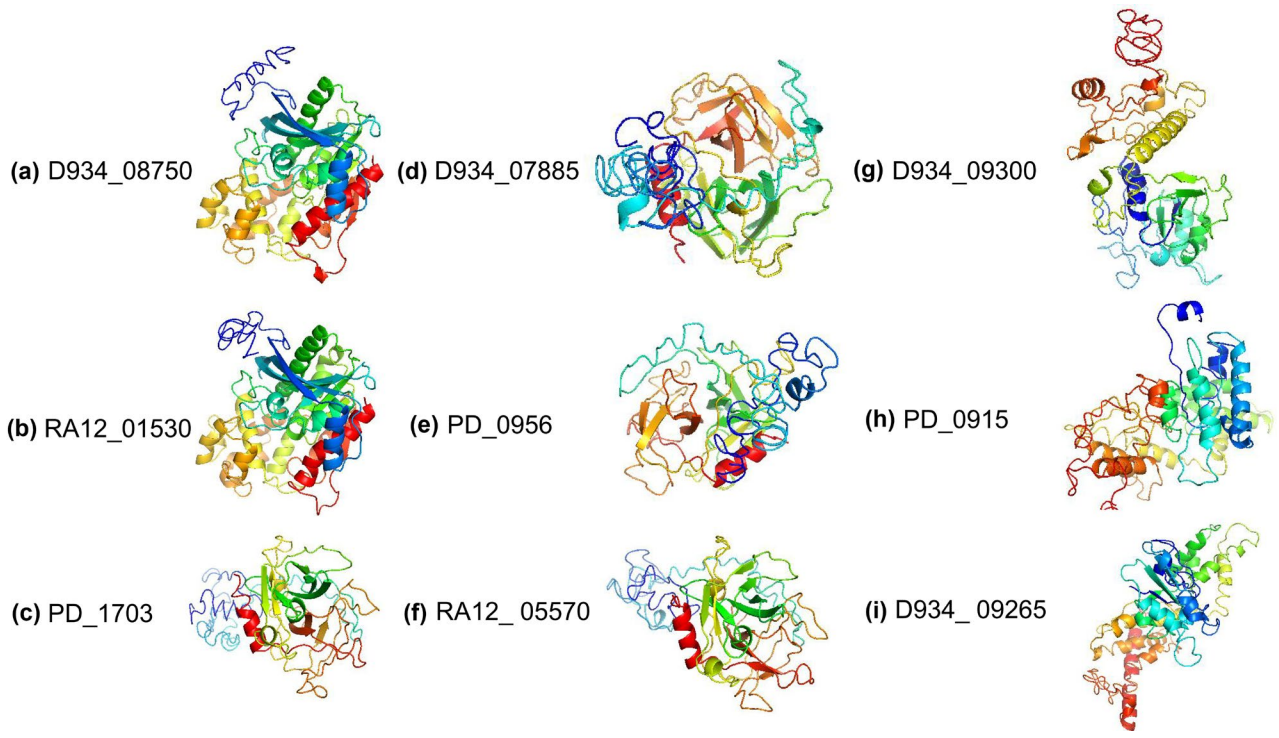

FIGURE 1 Predicted model presentation of the selected *Xylella fastidiosa* (Xf) putative effectors using the I-TASSER online server. The proteins presented here successfully elicited programmed cell death (PCD) in at least one plant cultivar/species tested. The colours suggest the protein orientation (blue: N-termini, red: C-termini). (a, b) Hydrolase/esterase (LipA), (c–f) hydrolase, and (g–i) zonula occludens toxin, according to their sequence similarities. We used Pymol v. 2.3.1 to visualize the structures (Schrodinger & DeLano, 2020)

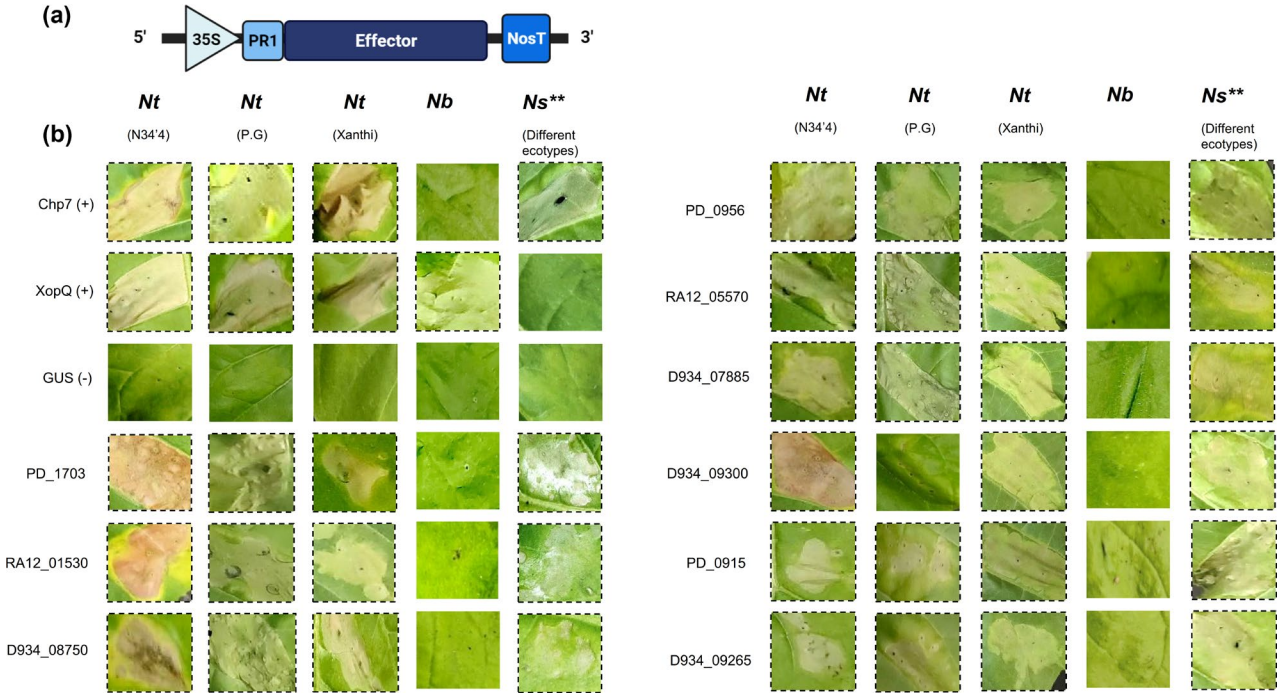

FIGURE 2 Putative *Xylella fastidiosa* (Xf) apoplastic effector proteins elicit programmed cell death (PCD) in *Nicotiana* species. (a) Schematic representation of the cassette that was cloned in an *Agrobacterium*-mediated plant transient expression system. Genes of interest were under the control of the constitutive CaMV 35S promoter. Secretion of the protein into the leaf apoplast was achieved through fusion to the secretion peptide of tobacco PR1a, which is cleaved during protein secretion (Lu et al., 2015). This figure was created with BioRender.com. (b) The apoplastic effector PR1 sp-Chp7 from *Clavibacter michiganensis* and the intracellularly acting effector XopQ from *Xanthomonas campestris* pv. *vesicatoria* were used as positive PCD hypersensitive response markers, while β-glucuronidase (GUS) served as the negative control for these assays (Adlung et al., 2016; Lu et al., 2015). Xf virulence factors that induced PCD following overexpression in the plant apoplast are illustrated above. PCD occurred 4 days post-infiltration at room temperature in all studied cases. The assays were repeated at least five times for each putative effector with similar results. **PD_1703, D934_08750, and RA12_01530 induced PCD in *Nicotiana sylvestris* ecotypes TW_136 and N_106 but not in ITB_626. PD_0956, D934_07885, and RA12_05570 induced PCD in *N. sylvestris* ecotypes A_34750352 but not in A_04750326. PD_0915, D934_09300, and D934_09265 induced PCD in *N. sylvestris* ecotype NS_25 but not in ITB_626

Cell death phenotypes varied in severity and seemed to develop at different rates. In order to comprehensively evaluate our results, we first assigned a cell death score to each observed necrotic phenotype based on its intensity (Figure S5). We then reviewed the frequency of a certain score regarding both the studied protein and the plant cultivar used in each experiment (Figure S5). For this, we have to consider potential differences in the transformation efficiency of distinct cultivars when using the transient expression system we applied. However, these results could also indicate that the same protein may elicit PCD of varying intensity when introduced into different *Nicotiana* relatives or cultivars of the same plant species.

The in silico structural prediction presented here indicates that all three proteins encoded by PD_0956, RA12_05570, and D934_07885, which successfully elicited PCD in all studied tobacco cultivars and in *N. sylvestris* ecotype A_34750352, have a high structural similarity with hydrolases (Figure 1; Table 1). Hydrolases form a big distinct enzyme class that includes enzymes which act as biochemical catalysts by using water molecules to break chemical bonds (Simon & Cravatt, 2010). This class contains enzymes classified as lipases, phosphatases, glycosidases, peptidases, and nucleosidases. Specifically, serine proteases/endopeptidases/hydrolases are enzymes where the nucleophilic serine residue in their active centre is used for the hydrolysis of their substrates (Simon & Cravatt, 2010). Hydrolases include proteases that are secreted by various pathogens having a wide range of functions in virulence (Simon & Cravatt, 2010). They also constitute an important group of Xf proteins including CWDEs (Nascimento et al., 2016). The presence of CWDEs in the apoplast can trigger immune responses, mostly through a modified "self"-recognition of degradation products of these enzymes by plant PRRs (van der Burgh & Joosten, 2019). Similarly, serine proteases delivered by pathogens into the apoplast have been shown to activate PCD (Lu et al., 2015). Provided that the putative enzymatic activity of PD_0956, RA12_05570, and D934_07885 is valid, their ability to elicit PCD could be considered a DAMP recognition event. Serine proteases are also present in large families of plant extracellular proteins that are often involved in signalling pathways associated with pathogen resistance (Hou et al., 2019). Therefore, manipulation of such pathways by bacterial proteases could be a virulence strategy.

Another prominent group of Xf proteins is that of PD_1703, RA12_01530, and D934_08750, which all trigger PCD in three tobacco cultivars and two *N. sylvestris* ecotypes, namely, TW_136 and NS_25, but not ITB_626 (Figure 2b) (Zhang et al., 2015). According

TABLE 1 Sequence similarity and structural template proteins for the predicted structures of the 19 selected *Xylella fastidiosa* putative proteins using I-TASSER

Xylella fastidiosa protein	Predicted protein size (Da)	Sequence similarity (PDB)	Description	Structural template (PDB)	Description
PD_0956	37,230.84	3WY8	Hydrolase/protease	3WY8	Hydrolase/serine protease
PD_0915	43,688.77	2R2A	Zonula occludens toxin (Zot)	2DHR	ATP-dependent metalloprotease/hydrolase
PD_1703	42,446.96	3WY8	Hydrolase/serine protease	1Z8G	Hydrolase/hydrolase inhibitor
D934_08750	46,789.42	3H2K	Hydrolase/esterase (LipA)	3H2K	Hydrolase/esterase (LipA)
D934_07885	37,308.92	3WY8	Hydrolase/serine protease	1Z8G	Hydrolase/hydrolase inhibitor
D934_09300	38,955.82	2R2A	Zonula occludens toxin (Zot)	2R2A	Zonula occludens toxin (Zot)
D934_09265	42,347.36	2R2A	Zonula occludens toxin (Zot)	4WWO	Transferase/transferase inhibitor
RA12_01530	46,084.80	3H2K	Hydrolase/esterase (LipA)	3H2K	Hydrolase/esterase (LipA)
RA12_05570	37,282.85	3WY8	Hydrolase/serine protease	1Z8G	Hydrolase/hydrolase inhibitor
D934_00810	98,061.87	5N8P	Membrane protein	3JAV	Transport protein
D934_05685	132,683.94	5N8P	Membrane protein	5IJO	Transport protein
D934_12725	96,871.81	3JAV	Transport protein	3JAV	Transport protein
D934_08755	47,285.77	3H2K	Hydrolase/esterase (LipA)	3H2K	Hydrolase/esterase (LipA)
D934_12535	46,472.88	3H2K	Hydrolase/esterase (LipA)	3H2K	Hydrolase/esterase (LipA)
D934_12795	40,907.20	7KVE	Blood clotting	1RWR	Cell adhesion
RA12_11155	17,962.85	4UIC	Sugar binding protein	1G6O	Hydrolase
RA12_11125	12,837.61	3V05	Toxin	5N8P	Membrane protein
RA12_03930	32,569.60	6VDP	Oxidoreductase	6VDP	Oxidoreductase
RA12_03905	21,817.80	6W1S	Gene regulation	6WIS	Gene regulation

to our structural analysis (Table 1), the last two proteins revealed similarities to LipA, a known *Xanthomonas oryzae* pv. *oryzae* CWDE, while PD_1703, even though it was previously characterized as a LipA-like protein (Nascimento et al., 2016), revealed similarities to hydrolase/serine proteases according to our I-TASSER structural prediction (Table 1). However, the hydrolase class is of the largest and most diverse enzyme families, which includes proteases and lipases, among others. Therefore, this might be a misannotation of the particular protein database.

LipA homologues are present in all sequenced xanthomonads and are predicted lipases, although LipA actually exhibits esterase activity (Apama et al., 2009) (Figure 3). LipA is known to elicit immune responses in rice and recent findings point to the possible involvement of the rice wall-associated kinase (WAK) OsWAKL21.2 in LipA recognition (Jha et al., 2007; Malukani et al., 2020). Structural similarities of LipA with PD_1703, RA12_01530, and D934_08750 suggest that these proteins act and are recognized in a similar manner (this finding is under further investigation by our group). Notably, PD_1703 has been shown to elicit PCD in grapevine, a known Xf Temecula-1 host. However, PD_1703 was found to be vital for Xf virulence in grapevines, suggesting that other virulence components of the pathogen could potentially suppress the PCD induction (Nascimento et al., 2016).

In this study we also focused on three Xf proteins, encoded by PD_0915, D934_09265, and D934_09300, which were found to elicit apoplastic PCD in *N. tabacum* and one *N. sylvestris* ecotype (Figure 2b; Table 1). Structural analysis revealed that this group consists of proteins with sequence and structural similarity to zonula occludens toxins (Zot proteins), although PD_0915 was predicted to be more confidently similar to a metal transferase. The Zot protein was described first in *Vibrio cholera*, where it is involved in intestinal barrier disturbance; however, Zot proteins were identified later in several other pathogens (Pérez-Reytor et al., 2018, 2020; Figure 3). Zot proteins have been associated with high cytotoxicity (Pérez-Reytor et al., 2018), though this is not always the case. For instance, in *Vibrio parahaemolyticus*, Zot expression did not positively correlate with cytotoxicity, but rather with actin disturbance, in infected cells (Pérez-Reytor et al., 2020). Putative Xf Zot proteins studied here appear not to be correlated with cytotoxic effects. Interestingly, the protein encoded by D934_09300 did elicit PCD in the apoplast of *N. tabacum* cultivars N34'4 and Xanthi and *N. sylvestris* ecotype NS_25, but this kind of response was not observed in *N. tabacum* cultivar Petit Gerard or in *N. sylvestris* ecotype ITB_626 and in *N. benthamiana* (Figure 2b). These data suggest specific recognition of D934_09300 and highlight the complexity of the plant surveillance system and its possible differentiation among distinct cultivars of the same species.

Finally, 10 putative Xf effectors that were unable to induce necrosis during in planta assays in the selected hosts were also analysed for their tertiary structures using the I-TASSER online server. Certain predictions could be made for their folding and function (Figure S1; Table 1). Notably, LipA-like proteins D934_08755 and D934_12535,

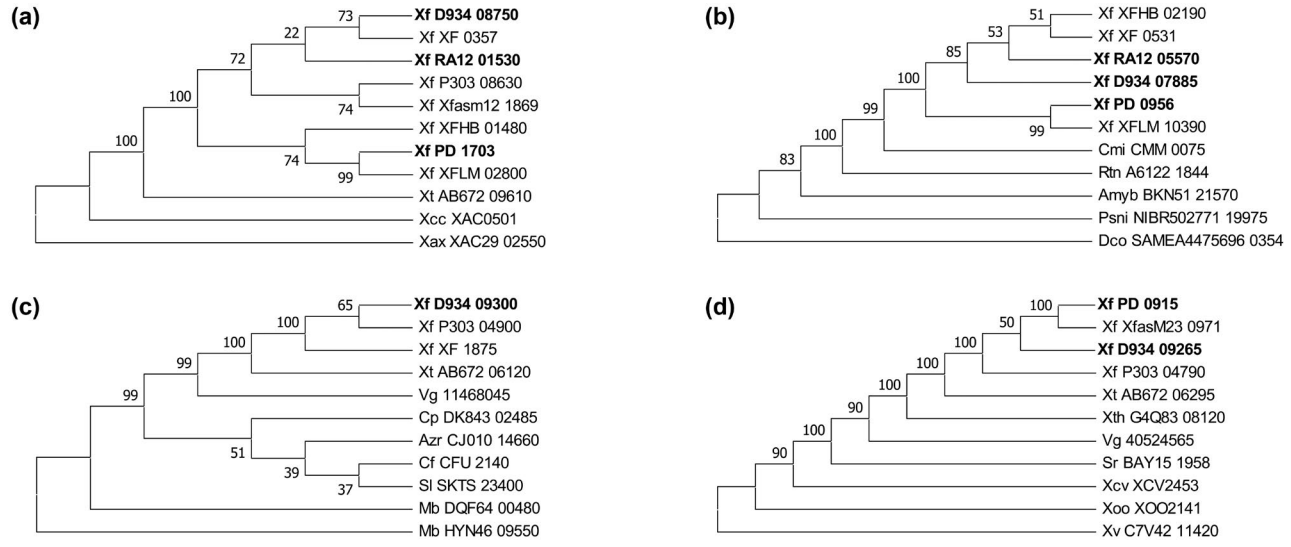

FIGURE 3 Phylogenetic trees were constructed for all 19 putative *Xylella fastidiosa* (Xf) effector proteins that are presented in this study, which were divided into subgroups based on their ability to elicit programmed cell death (PCD) and on their orthology, according to the KEGG database. (a–d) Proteins that induced PCD in this study, with a predicted orthology of (a) lipases, (b) peptidases, and (c, d) zona occludens toxins. These proteins were correlated with 35 close protein relatives from genera *Xanthomonas*, *Clavibacter*, *Ralstonia*, *Amycolatopsis*, *Pseudarthrobacter*, *Dermatophilus*, *Streptomyces*, *Stenotrophomonas*, *Moraxela*, *Azoarcus*, *Collimonas*, *Sulfurimicrobium*, and *Chromobacterium* and viruses Stenotrophomonas phage phiSHP2 and Stenotrophomonas phage SMA6. The evolutionary history in each group presented here was inferred using the neighbour-joining method (Saitou & Nei, 1987). The bootstrap consensus tree inferred from 1500 replicates is taken to represent the evolutionary history of the different taxa, based on amino acid sequences, as mentioned before. The evolutionary distances were computed using the Poisson correction method (Zuckerkandl & Pauling, 1965) and are in the units of the number of amino acid substitutions per site. Evolutionary analysis was conducted in MEGA X (Kumar et al., 2018). The abbreviations of microbes and the gene loci used for the construction of these phylogenetic trees are presented in Table S2

despite their strong correlation with other cell death inducers described in this study (PD_1703, D934_08750, RA12_01530), were incapable of causing similar phenotypes when expressed in the apoplast of *Nicotiana* species. This potentially indicates putative alterations to their active sites that prevent their binding to specific substrates of the plant cell wall.

All the 19 proteins were used for phylogenetic analysis using homologous proteins obtained from the KEGG database (Figure 3; Figure S6).

Our data collectively pinpoint nine proteins belonging to the sparsely studied Xf putative effectorome that can elicit PCD when transiently expressed and secreted into the leaf apoplast of different *Nicotiana* species. These proteins are structurally predicted as putative CWDEs or Zot proteins that originate from different Xf strains. The lack of signs of cytotoxicity, along with the predicted enzymatic activity of these proteins, hints at their possible recognition by the plant innate immunity system. At least in one case, the protein eliciting the response is a known required virulence factor of the pathogen, suggesting that it employs other virulence strategies to suppress immune responses and avoid recognition. The suppression of immune responses through T3SS-delivered effector proteins is a common feature among other members of the *Xanthomonadaceae* family (Jha et al., 2007). However, because Xf lacks such a system (Rapicavoli et al., 2018), how this bacterium avoids recognition by the host's surveillance system remains to be elucidated. In summary, our findings open up new research possibilities and encourage further investigation and identification of the related PRRs that could be a potential biotechnological tool to confer broad-spectrum disease resistance against Xf. Quite recently, the expression of a PRR receptor in sweet orange was reported to confer ligand-dependent activation of defence responses against a citrus-infecting strain of Xf (Mitre et al., 2021).

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AUTHORS' CONTRIBUTIONS

P.F.S. designed the research. M.S., K.K., D.T., and G.M. performed the research. V.N. and P.F.S. analysed the data. V.N. and A.D.F. provided technical support, laboratory material, and tools. M.S., K.K., D.T., and P.F.S. wrote the paper. All authors have read and approved the manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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