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REVIEW: PART OF A SPECIAL ISSUE ON PLANT IMMUNITY

PAMPs, PRRs, effectors and R-genes associated with citrus–pathogen interactions

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• **Background** Recent application of molecular-based technologies has considerably advanced our understanding of complex processes in plant–pathogen interactions and their key components such as PAMPs, PRRs, effectors and R-genes. To develop novel control strategies for disease prevention in citrus, it is essential to expand and consolidate our knowledge of the molecular interaction of citrus plants with their pathogens.
• **Scope** This review provides an overview of our understanding of citrus plant immunity, focusing on the molecular mechanisms involved in the interactions with viruses, bacteria, fungi, oomycetes and vectors related to the following diseases: tristeza, psorosis, citrus variegated chlorosis, citrus canker, huanglongbing, brown spot, post-bloom, anthracnose, gummosis and citrus root rot.

Key words: Citrus immunity, citrus psorosis, tristeza of citrus, citrus variegated chlorosis (CVC), citrus canker, huanglongbing (HLB), brown spot, post-bloom, anthracnose, gummosis, and citrus root rot.

INTRODUCTION

Citrus trees are grown in more than 140 countries in tropical and subtropical areas. The long life span of a citrus tree (in some cases more than a century) leads to complex interactions with micro-organisms throughout the soil and above-ground areas (Wang *et al*., 2015). Citrus production is heavily affected by diseases caused by a diverse range of viruses, bacteria, fungi, oomycetes and nematodes. These diseases increase the cost of production, are responsible for low productivity and involve annual losses of millions of dollars (Neves *et al*., 2014). In this context, it is essential to expand and consolidate knowledge of citrus interactions with their pathogens to implement new control and/or management approaches. The advancement of molecular biology technologies has greatly expanded our understanding of the complex processes of host–pathogen interactions, both in model herbaceous as well as in woody and perennial plants such as citrus species. The complexity of citrus pathosystems, associated with extensive monospecific or monoclonal plantations, indicates that the chemical control model for disease, pests or vectors, when feasible, cannot be sustainable in the medium and long term.

Knowledge of the biological processes that lead to disease and of how organisms behave during the interactions with their hosts is crucial for proposing new control strategy models. This review provides an update on important aspects of the interaction of citrus with several of their pathogens. Whilst considerable advances have been made, it is also clear that many stages of disease development processes are still not well understood and behave differently than observed in model plant systems.

GENERAL ASPECTS OF PLANT INNATE IMMUNITY

Plant cells have a large number of receptors anchored on the cell surface, which are crucial to sense extracellular signals and for cell-to-cell communication. Pattern recognition receptors (PRRs) act as cellular 'antenna' and allow plants to detect a wide range of danger signals including non-self (PAMPs, MAMPs, HAMPs and VAMPs – pathogen, microbe, herbivore and virus-associated molecular patterns) and even self-derived compounds (damage-associated molecular patters or DAMPs), which are released upon herbivore and pathogen attack. The presence of PRRs represents a critical step in host perception and self-defence against attackers by triggering innate immune responses (Jones and Dangl, 2006).

The structures perceived by PRRs are conserved across certain microbe classes and are related to primary functions for their fitness (Medzhitov and Janeway, 1997; Nürnberger and Brunner, 2002). Thus, the genetic factors coding for the recognized pattern molecules are less likely to be mutated or lost during the microbe evolutionary processes. The recognition by PRRs of this set of conserved molecules confers broad-spectrum resistance against microbes sharing the same PAMP. Despite this conservation, some PAMPs are still subject to selection pressure during co-evolution with host plants.

The modification of key amino acid residues at the recognition sites allows adapted pathogens to evade perception by PRRs (Boller and Felix, 2009; Monaghan and Zipfel, 2012).

Pattern recognition receptors

Although only a few PRR–PAMP pairs have been identified, all the known PRRs are modular transmembrane proteins and they are either receptor-like kinases (RLKs) or receptor-like proteins (RLPs) containing ligand-binding ectodomains (Goff and Ramonell, 2007; Monaghan and Zipfel, 2012). In general, plant RLKs have a single pass transmembrane (TM) domain for anchorage, a variable N-terminal extracellular domain (ECD) for ligand binding and a C-terminal intracellular kinase domain (KD) that relays downstream signalling (Shiu and Bleecker, 2001). RLKs represent a large and diverse gene family with more than 600 and 1100 members identified in *Arabidopsis* and rice, respectively (Shiu and Bleecker, 2001; Shiu *et al*., 2004). The RLKs with leucine-rich repeats as ECDs (LRR–RLKs) constitute the largest subfamily and contain most of the identified PRRs (Goff and Ramonell, 2007). The LRR–RLKs Flagellin Sensing 2 (FLS2) and EF-Tu Receptor (EFR) from *Arabidopsis* and XA21 from rice represent the best-studied plant PRRs. FSL2 and EFR activate PAMP-triggered immunity (PTI) responses by sensing elicitor epitopes from bacterial flagellin (flg22), elongation factor Tu (elf18), whereas XA21 is elicited by Ax21 (sulfated RaxX) (Boller and Felix, 2009; Pruitt *et al*., 2015). The *Arabidopsis* PEPR1 and PEPR2 are LRR–RLKs that trigger PTI defence responses by perceiving as DAMPs the conserved Pep epitopes produced by cleavage of propeptides (PROPEPs) (Yamaguchi and Huffaker, 2011). Although RNA-silencing represents the main resistance strategy against viruses, *Arabidopsis* NIK1 and NIK2 are LRR–RLKs with an important role in antiviral immunity responses. Instead of LRRs, some RLKs perceive PAMPs by LysM motifs in the ECD (LysM–RLKs), such as CERK1 (Chitin Elicitor Receptor Kinase 1) that has three extracellular LysM domains and triggers PTI by recognizing fungal chitin oligosaccharides (Miya *et al*., 2007). Plant RLPs also have a TM domain and an ECD but do not have a KD, except for a short cytoplasmic tail lacking any obvious signalling domain. Thus, it must complex with KD proteins to transduce the signals in the cytoplasm after PAMP recognition by the ECD (Shiu and Bleecker, 2003). The *Arabidopsis* RLPs LYM1 and LYM3 and the rice orthologues LYP4 and LYP6 recognize peptidoglycans as PAMPs but complexes with LysM RLKs are necessary to trigger immunity responses (Zipfel, 2014). Other important PRR RLPs identified are the rice chitin elicitor-binding protein (CEBiP), which recognizes fungal chitin as an elicitor, and the tomato LeEIX1 and LeEIX2, which are able to detect fungal ethylene inducing xylanase EIX as PAMP (Kaku *et al*., 2006).

PAMP-triggered immunity (PTI)

To activate the PTI response, pathogen structures must be perceived by the PRR ECD, with subsequent signal transduction in the cytoplasm. Several molecules are used by plants to encode signals acquired by pathogen recognition for delivery of information downstream of PRRs to proteins related to signal interpretation and activation of defence response genes (Zipfel *et al*., 2004; Denoux *et al*., 2008). PAMP recognition leads to numerous plant signals, including an oxidative burst by the generation of reactive oxygen species (ROS), calcium influx, activation of the mitogen-activated protein kinase (MAPK) cascade, nitric oxide (NO) burst, ethylene production, callose deposition at the cell wall, and expression of defence-related genes involved in immunity responses (Boller and Felix, 2009).

GENERAL ASPECTS OF EFFECTOR-TRIGGERED IMMUNITY

Effectors

Many definitions of effectors are available in the literature. For this review, the following definition will be used: effectors are molecules released/associated with an organism that alters the physiology, structure or function of another organism. Specifically, effectors are pathogen molecules that can modify host cell structures and manipulate function, facilitating infection and/or triggering defence responses. Unlike the terms ‘avirulence’, ‘elicitor’, ‘toxin’ and ‘virulence’, the term effector is neutral and does not imply a negative or positive impact on the outcome of the host–pathogen interaction. Effectors are responsible for promoting pathogen penetration and persistence inside the host tissue, as well as suppression of immune responses, allowing access to nutrients, proliferation and growth (Göhre and Robatzek, 2008).

Common features from well-characterized effectors are used by plant pathologists to search for possible candidate molecules from new and old pathogens. These candidates are usually small secreted proteins, which are rich in cysteine and show no obvious homology to other known proteins (Göhre and Robatzek, 2008). Secreted effectors reach their cellular target either at the intercellular interface of the host and pathogen cells (apoplastic effectors) or inside the host cells (cytoplasmic effectors) (Kamoun, 2006; Djamei *et al*., 2011).

R-genes

Plant defence through effector-triggered immunity (ETI) is based on the highly specific interaction between products from pathogen avirulence genes (Avr) and products from host resistance genes (R), according to the gene-for-gene hypothesis (Flor, 1971). R proteins can recognize pathogen effectors directly or indirectly through their effects on host cells (Win *et al*., 2012). Indirect recognition occurs through R protein-mediated monitoring of effector disturbances in distinct host cellular targets of an effector, consistent with the so-called ‘guard hypothesis’ (Dangl and Jones, 2001). Currently, two variations of this model are recognized. In one, the R receptor is constitutively associated with the host intermediate factor, whereas in the other, the pathogen effector first associates with a host target and the complex formed is then recognized by the immune receptor (Caplan *et al*., 2008; Elmore *et al*., 2011). The major evidence for the guard hypothesis was obtained in the R/Avr system between *Arabidopsis thaliana* and *Pseudomonas syringae* pv. tomato, where the modification

of the host factor RIN4 by the bacterial Avr gene product activates the R protein RPM1, resulting in plant resistance (Mackey *et al*., 2002).

Structurally, R-genes commonly present a central nucleotide-binding site (NBS) domain, a C-terminal LRR region to mediate pathogen recognition and an N-terminal variable domain mainly identified as TIR (Toll/Interleucina-1) or CC (Coiled-coil) (Elmore *et al*., 2011; Gururani *et al*., 2012). Besides TIR-NBS-LRR and CC-NBS-LRR, other major classes of R-genes include the RLKs (containing an extracellular LRR, a transmembrane domain and a cytoplasmic kinase domain), RLPs (which are similar to the RLKs but lack the kinase domain) and cytoplasmic enzymatic R-genes that contain neither LRR nor NBS groups (Gururani *et al*., 2012).

CITRUS–PATHOGEN INTERACTIONS

Citrus overview

The Brazilian citrus industry is one of the most important in the world. It accounts for over a third of the world's sweet orange production and more than 50 % of the orange juice production, both frozen-concentrated orange juice (FCOJ) and not from concentrate (NFC) juice. São Paulo State is the main producer, processor and exporter of orange juice, and accounts for 80 % of the orange, lime and lemon production and 45 % of the mandarin production in Brazil. From 2005 to 2015, São Paulo was responsible for the production of 97 % of FCOJ, 99·5 % of NFC juice, 95 % of essential oils, and 99·5 % of dried or fresh orange fruit exports in Brazil (Neves *et al*., 2014).

Although Brazil is the world's largest producer of fresh oranges and orange juice, productivity of the Brazilian citrus industry is considered very low (approximately two boxes per tree per year, each box with 40·8 kg). Low yield is associated with high incidence of pests and diseases, reduced genetic diversity of scions and rootstocks, and production in non-irrigated areas.

It has been estimated that more than 60 % of the costs of citrus production in Brazil are associated with control of pests and diseases. Diseases such as citrus variegated chlorosis (CVC), leprosis, black and brown spot (CBS), sudden death, citrus canker, gummosis, root rot, tristeza and huanglongbing (HLB) are the more important diseases seen in orchards (Machado *et al*., 2011).

When a disease is associated with rootstock, its replacement by more resistant material has been the most effective method of control. However, when the disease mainly affects the canopy, replacement with a more resistant cultivar is not always feasible, either because there is no available resistance source or the resistant cultivar is not acceptable to the market (Machado *et al*., 2011). Moreover, the complexity of citrus pathosystems is very high and often involves pathogens that invade plants systemically and with highly efficient vectors. Genetic improvement-based control is possible, but involves long and costly selection and evaluation programmes, with genetic barriers that can hinder breeding.

Pattern recognition receptors in citrus

Despite the large number of disease-causing pathogens in citrus, no PRR has yet been functionally well characterized. However, some RLKs and RLPs have been identified with roles in pathogen perception in innate immunity responses (Fig. 1). Rodrigues *et al*. (2013) analysed RNA-Seq data for CVC in resistant mandarin (*Citrus reticulata*), infected with *Xylella fastidiosa*. They identified, in addition to a leucine-rich repeat receptor-like protein (RLP12), two other up-regulated genes (Ciclev10004108m and Ciclev10014130m) similar to LRR-RLKs, which might be related to PTI responses activated by *X. fastidiosa* PAMP recognition. Moreover, a novel RLK with a lectin domain (Lec) in the ECD was isolated from *C. limon* in response to the fungus *Capnodium citri*, the causal agent of sooty mould. Investigations of this interaction suggest a defence mechanism against pathogens mediated via a signal transduction pathway which can be modulated by a PRR (De Felice and Wilson, 2009).

Transient expression of the flagellin- and hook-associated protein (Fla) from the bacterium *Candidatus* Liberibacter asiaticus (*Ca*Las), which causes HLB citrus disease, induced cell death, callose deposition and up-regulation of BAK1 transcripts in *Nicotiana benthamiana*. The conserved domain $flg22_{Las}$ also triggered different degrees of PAMP activity in citrus plants, suggesting that Fla_{Las} acts as a PAMP and can be recognized in citrus in addition to *N. benthamiana* (Zou *et al*., 2012). The conserved flg22 derived from *Xanthomonas citri* subsp. *citri*, the bacterial agent of citrus canker disease, also triggered rapid ROS production and induction of PTI marker genes, mainly in the group of more resistant citrus genotypes (Shi *et al*., 2015). These results suggest a citrus PRR that is able to recognize flagellin exists and might be important in triggering resistance against HLB and *X. citri* subsp. *citri*. Functional orthologues of FLS2 with different perception specificities were previously characterized in other plants such as tomato, rice, grapevine and *N. benthamiana* (Robatzek *et al*., 2007; Takai *et al*., 2008; Chakravarthy *et al*., 2010). Genetically engineered plants with overexpression of PRRs provide a promising strategy to increase plant immunity. Transgenic *C. sinensis* overexpressing rice Xa21 showed increased resistance to citrus canker. Recently, a reduced susceptibility to *X. citri* was also demonstrated in citrus plants expressing the FLS2 receptor from *N. benthamiana* (Hao *et al*., 2016).

The Citrus EST project (CitEST) consists of an expressed sequence tag (EST) database obtained from citrus species under a diverse range of conditions, including stresses caused by the main pathogens (Targon *et al*., 2007). This database represents a useful source of genomic information for further understanding of host defence mechanisms such as those associated with innate immunity responses. Guidetti-Gonzalez and Carrer (2007) conducted *in silico* analyses with CitEST and identified genes with similarity to the RLP ethylene-inducing xylanase EIX1 in *C. sinensis* infected with *X. fastidiosa*, suggesting a specific role against bacterial pathogens rather than associated with fungal response. Moreover, contigs and singletons similar to Xa21 and Xa26 rice receptors were identified in *C. sinensis*, *C. reticulata* and *Poncirus trifoliata* in response to *X. fastidiosa* and *Citrus tristeza virus* (CTV). Functional characterization of the available data from CitEST is necessary to advance our

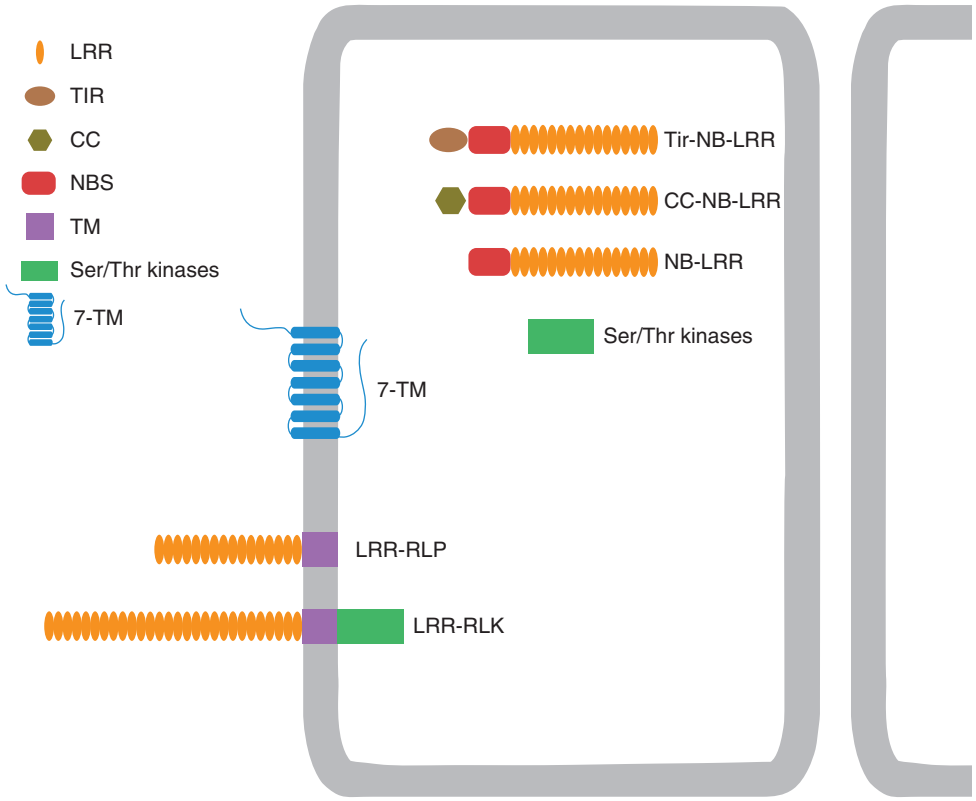

Fig. 1. Domain structures of *Citrus* PRRs and R-genes.

understanding of the mechanisms associated with citrus innate immunity responses.

R-genes in citrus

A recent genome-wide comparative analysis identified NBS genes in the genomes of *C. sinensis* and *C. clementine* (Y. Wang *et al*., 2015). The authors found 618, 650 and 508 NBS genes in *C. clementina*, *C sinensis* China and *C. sinensis* USA, respectively. However, the typical TIR- and CC-NBS-LRR classes of R-genes correspond to only 32 % of the total NBS genes in *C. clementina*, 29 % in *C. sinensis* China and 18 % in *C. sinensis* USA genomes. Phylogenetic analysis discriminated *Citrus* NBS genes into three groups with highly variable C-terminal LRR motifs, responsible for recognizing pathogen effectors, which implies different roles for the groups in the citrus immune system. The majority of *Citrus* NBS genes are physically clustered in the genome. Most clusters contain genes from the same phylogenetic group; genes in the same cluster tend to be on the same strand, which indicates that the expansion of NBS genes in *Citrus* is primarily due to tandem duplication. Furthermore, both the hybrid *C. sinensis* and the original *C. clementina* have similar numbers and types of NBS genes, consistent with their derivation from a common ancestor.

Besides this screening at the genome level, a first attempt to identify genes coding for resistance proteins in the citrus transcriptome was accomplished using CitEST. In the CitEST database, 259 contigs and 332 singletons related to R-genes were identified, wherein a total of 137 R-genes showed similarities to different categories including NBS-LRR, CC-NBS-LRR, TIR-NBS-LRR, cytoplasmic Ser/Thr kinases and the seven-transmembrane (7-TM) family of resistance proteins. Although some of those sequences were present in citrus libraries from healthy and infected samples, most came from plants challenged with pathogens. The large number of expressed putative R-like genes found in the CitEST database, mainly in pathogen-infected libraries, suggests that they function as resistance genes in citrus (Guidetti-Gonzalez and Carrer, 2007). The structures of these R-genes are shown in Fig. 1.

Other evidence for the involvement of R-genes in the citrus defence response to pathogens comes from global gene expression analyses. In CVC-resistant mandarins (*C. reticulata* Blanco), one gene encoding an NBS-LRR-like disease resistance protein was up-regulated 30 d after inoculation with *X. fastidiosa*, indicating that some bacterial signals are recognized by the plant, triggering defence mechanisms to prevent disease (Souza *et al*., 2007). Furthermore, the CC-NBS-LRR gene was up-regulated in mandarin 1 d after infection with *X. fastidiosa* (Rodrigues *et al*., 2013). Likewise, in citrus hybrids resistant to *Phytophthora parasitica* infection, both the TIR-NBS-LRR RPS4 gene and another R-gene of the same class were up-regulated, indicating that these genes may be involved in the recognition of effectors produced by *P. parasitica*, thus inducing the plant defence system (Boava *et al*., 2011).

Finally, some citrus R-genes were also functionally validated, such as the Ctv-R, a CC-NBS-LRR gene, which confers resistance to CTV in *P. trifoliata*. Ctv-R incorporation into susceptible plants results in different levels of resistance to CTV infection, confirming its role as a disease resistance protein (Rai, 2006). It remains to be demonstrated whether the large number of putative *Citrus* R-genes and their allelic variants identified in both genome and transcriptome studies effectively promote defence in resistant plants against the wide range of citrus pathogens.

CITRUS–VIRUS INTERACTIONS

In contrast to other plant–pathogen systems, the primary plant immune strategy against viruses is RNA silencing (Incarbone and Dunoyer, 2013), a mechanism for control of both gene expression and viral infection mediated by the action of small interfering RNAs (siRNAs). Antiviral RNA silencing is triggered by double-stranded RNA (dsRNA) replication intermediates or structures within RNA viral genomes. These viral dsRNAs are recognized by the RNAseIII endonuclease Dicer (DCL) that processes them into virus-derived siRNAs. A strand of these molecules is incorporated into an RNA-induced silencing complex (RISC) containing an Argonaute (AGO) protein, which finally guides sequence-specific silencing of the homologous viral genome. This mechanism of antiviral control is highly efficient, since the target sequence is dictated by the virus itself, which in turn cannot evolve to avoid sequence-based recognition (Obbard *et al*., 2009). However, viruses have developed a means to counteract the RNA silencing: viral suppressors of RNA silencing (VSRs). The VSRs are present in most, if not all, plant viruses, presenting different modes of action to target many steps of the RNA silencing pathway (Incarbone and Dunoyer, 2013).

A remarkable parallel can be observed between the activation and suppression of RNA silencing on the one hand and the classic zig-zag scheme for PTI/ETI resistance on the other (Fig. 2). In fact, it has been accepted that these processes are manifestations of the same phenomenon (Incarbone and Dunoyer, 2013). In this regard, virus-derived dsRNA can be considered a VAMP, because it constitutes a mandatory pattern in RNA virus replication. The silencing machinery, composed of DCL and RISC, forms the first line of defence that recognizes those patterns, similar to PTI. VSRs are the virulence effectors that overcome RNA silencing, triggering ETS. As expected for pathogen effectors, VSRs are highly diverse, involving many different strategies to overcome plant defence, and are under strong selection, evolving much faster than other viral genes (Obbard *et al*., 2009). As a consequence, plants could present R-proteins capable of perceiving VSR effects and triggering typical outputs of ETI, such as the hypersensitive response (HR) (Incarbone and Dunoyer, 2013).

Citrus–Citrus tristeza virus *interaction*

Our current understanding of the mechanisms involved in *Citrus*–virus interactions is mainly focused on CTV, a positive-sense single-stranded RNA (ssRNA) virus member of the genus *Closterovirus*. Depending on the virus isolate and the variety/

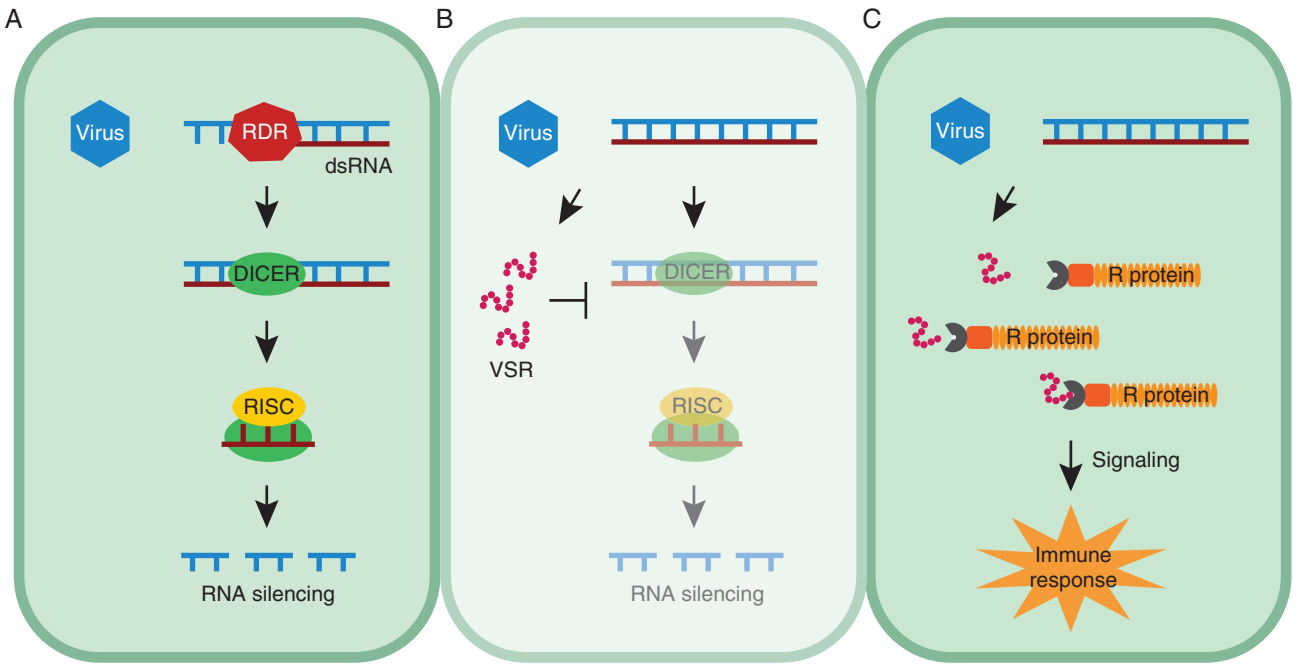

Fig. 2. Parallel between the classical pattern-triggered immunity (PTI)/effector-triggered immunity (ETI) response and the framework of RNA silencing activation and suppression. (A) Upon virus attack, RNA-dependent RNA polymerases (RDR) produce dsRNA, a virus-associated molecular pattern (VAMP). Similar to PTI, the RNA silencing machinery coordinated by Dicer and RNA induced silencing complex (RISC) recognize and process viral PAMPs, forming the first layer of defence. (B) Viruses have acquired viral supressors of RNA silencing (VSRs) as effectors that suppress host defence, resulting in effector-triggered susceptibility (ETS). (C) In turn, plants developed resistance (R) proteins that recognize viral effectors and activate ETI.

TABLE 1. *Effectors from citrus viruses and cognate R proteins from citrus species*

Virus	Effector	R protein	Reference
Citrus tristeza virus	p20	–	Lu *et al*. (2004)
	p23	–	
	Coat protein (CP)	–	
	p33	–	Tatineni and Dawson (2012)
	p18	–	
	p13	–	
	–	Ctv-R (Poncirus trifoliata)	Rai (2006)
Citrus psorosis virus	24K	–	Reyes *et al*. (2016)

rootstock combination, CTV can cause any of four distinct syndromes in citrus plants: decline (plant death), stem pitting (aberrant phloem development, resulting in pits in the wood), seedling yellows (stunting and leaf chlorosis) and, most commonly, a complete lack of symptoms, even when the virus multiplies to high titres (Dawson *et al*., 2013). In the last century, due to the massive use of the decline-sensitive sweet orange/sour graft orange combination, CTV caused losses of over 100 million trees worldwide, becoming the most economically important virus affecting the citrus industry (Moreno *et al*., 2008).

CTV infection is restricted to phloem-associated cells, resulting in limited cell-to-cell and long-distance movement. Host interference in both virus infection mechanisms is dependent on the citrus genotype (Dawson *et al*., 2013). The virus systemically infects its hosts using only the long-distance movement from source-to-sink, with cell-to-cell movement absent or limited to only small clusters of adjacent cells even in the more susceptible citrus species (Folimonova *et al*., 2008). This distribution within the host is thought to be related to the interaction of virus gene products with specific hosts (Dawson *et al*., 2013).

To reduce CTV titre and systemic infection, citrus species employ RNA silencing. In turn, CTV has developed three effectors that exhibit VSR activities: p20, p23 and coat protein (CP) (Lu *et al*., 2004). The p20 and CP proteins suppress intercellular silencing, preventing the spread of the silencing signal and probably the activation of host defences, while p20 and p23 suppress intracellular silencing, reducing viral degradation. Given that RNA silencing is a central host defence to contain viral replication and even restrict the virus to phloem cells, the constitutive expression of p23 has been reported to increase the CTV titre in sour orange and to allow CTV to escape from the confines of the phloem in both sour and sweet orange (Fagoaga *et al*., 2011). However, even with the establishment of a successful systemic infection, some degree of CTV genome silencing still occurs, suggesting that RNA silencing cannot completely inhibit viral replication and infection and that the three effectors cannot completely block RNA silencing. The constant arms race between the virus and its hosts (Obbard *et al*., 2009; Dawson *et al*., 2013) may have led to this balanced co-evolutionary process, so that the virus remains in the host without causing severe symptoms or plant death.

Besides the three effectors with VSR activities required for CTV to overcome host resistance, the virus has other non-conserved genes – p33, p18 and p13 – that are not needed for infection of most CTV hosts, but are necessary in different combinations for infection of certain citrus species (Tatineni *et al*., 2011). It has been suggested that CTV acquired these non-conserved genes for movement and overcoming host resistance, further extending its host range (Dawson *et al*., 2013).

Notably, the CTV effectors identified (Table 1) are not only required to suppress host defences and establish infection, but can also be involved in induction of disease symptoms. For example, the balance between expression of p33, p13 and p18 determines the severity of the stem-pitting symptom: deletion of different combinations of these genes can induce large increases in stem pits (Tatineni and Dawson, 2012). Similarly, ectopic expression of the VSR p23 induces virus-like symptoms (Flores *et al*., 2013). For other described pathosystems, it has been shown that ectopic expression of VSRs alters the plant small RNA regulatory pathway, inducing symptoms (Pacheco *et al*., 2012). Changes in the accumulation patterns of miRNAs have also been reported in CTV-infected citrus plants (Ruiz-Ruiz *et al*., 2011), suggesting that suppression of host RNA silencing defences by CTV also affects the plant small RNA regulatory pathway, resulting in symptom expression.

Although CTV effectors are known, no corresponding plant R-gene has been identified. However, a CC-NB-LRR R protein with an unknown corresponding Avr CTV gene has been characterized (Rai, 2006) (Table 1). The locus, Ctv-R, is a single dominant gene from *P. trifoliata*, which confers broad-spectrum resistance to the majority of CTV isolates. Sequence analysis of the Ctv genomic region located the locus in a 121-kb region comprising ten genes. Susceptible grapefruit plants transformed with some of these ten candidate Ctv-R genes result in different levels of resistance, such as an absence of initiation of infection, its slow spread or an initial appearance of infection followed by its subsequent eradication (Rai, 2006). However, some of the viral proteins recognized by NB-LRR are not VSRs (de Ronde *et al*., 2014). Therefore, the CTV protein recognized by CTV-R may not necessarily be analogous to other effector proteins that suppress PTI.

Citrus–Citrus psorosis virus *interaction*

Besides CTV, information on citrus molecular interactions with other infecting viruses is still scarce. However, the RNA silencing mechanism has been suggested to be involved in the citrus response to *Citrus psorosis virus* (CPsV), a negative-stranded RNA virus from the genus *Ophiovirus* and causal agent of psorosis disease (Achachi *et al*., 2014). In CPsV-infected plants, higher temperatures promote attenuated symptoms, reduce levels of viral RNA and increase virus-derived siRNA. Previous work revealed that RNA silencing is weaker

at low temperatures and stronger at high temperatures (Chellappan *et al*., 2005). Thus, the impairment of CPsV infection may be due to the temperature-induced enhancement of RNA silencing (Velázquez *et al*., 2010).

To date, a VSR from CPsV to counteract viral silencing has not been characterized. However, a recent study demonstrated that infection by CPsV promotes a down-regulation of *C. sinensis* endogenous micro-RNAs (miRNAs) (mainly miR156 and miR171) and a consequent up-regulation of its target genes (the transcription factors *Squamosa promoter-binding protein-like*, SLP, and *Scarecrow-like*, SCL) (Reyes *et al*., 2016). Modulation of the miRNA pathway by plant viruses as a result of VSR activities has already been described and is thought to be a viral strategy to bypass host defences and induce symptoms (Jay *et al*., 2011; Padmanabhan *et al*., 2013). The up-regulated targets, SPL and SCL, are involved in the activation of programmed cell death and in the decrease of chlorophyll biosynthesis, respectively; thus, large amounts of both transcripts may contribute to the necrosis and chlorosis symptoms manifested by citrus plants infected by CPsV (Reyes *et al*., 2016). Furthermore, the authors demonstrated that the CPsV 24K protein physically interacts with pre-miR156 and pre-miR171, suggesting the protein is responsible for altering precursor processing and subsequent biogenesis of miRNAs (Reyes *et al*., 2016). Once the 24K protein affects the miRNA pathway, whose components are shared by antiviral silencing, and induces the expression of genes probably involved in disease symptom development, the protein may be considered a potential VSR and effector of CPsV (Table 1).

CITRUS–BACTERIA INTERACTIONS

Citrus–Xylella fastidiosa *interaction*

Xylella fastidiosa is a Gram-negative bacterium that causes CVC disease in sweet orange and Pierce's disease (PD) in grapevine, and infects other economically important crops (Hopkins and Purcell, 2002; Bové and Ayres, 2007). *Xylella fastidiosa* is limited to xylem vessels and its transmission under field conditions occurs via insect vectors (sharpshooters). Once in the susceptible citrus plant, *X. fastidiosa* systemically colonizes the xylem vessels forming a biofilm. The resultant sap flow blockage in vessels by biofilm has been suggested as the main factor associated with *X. fastidiosa* pathogenicity. In *C. sinensis*, leaf symptoms are described as yellow spots on the adaxial surface that can develop into necrosis as the disease progresses (Souza *et al*., 2009).

In fruits, the disease promotes size reduction and premature ripening, which has been responsible for losses of millions of dollars in citrus agribusiness (Bové and Ayres, 2007).

Given the economic importance of this bacterium, several studies have been conducted to understand the biology of *X. fastidiosa*, including functional gene studies, and investigation of the mechanisms of tolerance to antimicrobial compounds and bacterial colonization (Rodrigues *et al*., 2008; Caserta *et al*., 2010; Muranaka *et al*., 2012). However, few studies have focused on the interaction of *X. fastidiosa* and citrus, especially regarding the role of effectors in disease development.

In *C. reticulata* (resistant to *X. fastidiosa*), Coletta-Filho *et al*. (2007) demonstrated that *X. fastidiosa* can survive in the initial stages of infection in this host, suggesting that the resistant plant recognizes *X. fastidiosa* in some way, and triggers the plant defence response. To better understand this interaction, EST libraries were created using sweet orange with and without CVC symptoms and mandarin inoculated with *X. fastidiosa* (Gmitter *et al*., 2012). Data analysis showed that genes associated with a defence response are also up-regulated in susceptible plants, but primarily when the bacteria have already colonized the plant, showing that these genes are induced but at later stages of infection. In contrast, in resistant plants, different sets of genes are up-regulated at different time points during the interaction. At initial stages of infection, the induced genes were related to pathogen recognition, signal transduction and defence. At the second time point, the induced genes were associated with signal transduction (MAPK cascade) and with a defence response, including ethylene-related transcription factor, LOX gene associated with the jasmonic acid pathway, and *S*-adenosyl-L-methionine: salicylic acid methyltransferase (Gmitter *et al*., 2012). These findings reinforce the hypothesis that the resistant host triggers defence genes after recognition of *X. fastidiosa*. Indeed, Rodrigues *et al*. (2013) verified by RNA-seq analysis that genes associated with PTI are induced in *C. reticulata* 1 d after *X. fastidiosa* inoculation. Such genes are putative PAMP receptors, genes associated among others with the formation of secondary xylem, cell-wall synthesis and ROS, suggesting that this plant was able to recognize unknown PAMPs of *X. fastidiosa* and, consequently, activated defence responses. Although no PAMPs for *X. fastidiosa* have yet been characterized, Kunze *et al*. (2004) demonstrated elicitation activity of EF-Tu peptide from *X. fastidiosa* in an alkalinization assay. However, the elicitation was much lower when compared with other peptides from different bacteria. Additional studies for verification that EF-Tu is a bona fide PAMP for *X. fastidiosa* are warranted.

Another gene induced identified following RNA-seq and EST analyses is a CC-NBS-LRR gene (Gmitter *et al*., 2012; Rodrigues *et al*., 2013). The CC-NBS-LRR genes have been reported as cytoplasmic receptor proteins, which usually recognize effector proteins triggering ETI. Although no effector has been described so far in the *X. fastidiosa* CVC strain, in the PD strain some putative effectors were recently reported (Zhang *et al*., 2015; Nascimento *et al*., 2016). Among them, the LipA/LesA gene (PD1703) (Zhang *et al*., 2015; Nascimento *et al*., 2016) was characterized as a lipase/esterase and was identified as a key gene for pathogenesis of *X. fastidiosa* in grapevine (Nascimento *et al*., 2016). A loss-of-function *lesA* mutant produced far fewer symptomatic leaves when compared with the wild-type infection. In the CVC strain there is a homologue of the LipA/LesA gene in its genome (XF0357) (Nascimento *et al*., 2016); however, whether this gene has a role in the pathogenesis of *X. fastidiosa* in citrus needs to be verified. These results suggest that *X. fastidiosa* might be secreting effectors by alternative systems. The type III secretion system (T3SS), which is classically associated with secretion of effectors in other bacteria, is lacking in *X. fastidiosa* strains. Thus, the CC-NBS-LRR protein could be recognizing some yet unknown effector and, consequently, leading to plant defence responses (Rodrigues *et al*., 2013).

Rodrigues *et al*. (2013) reported that the resistance mechanism of *C. reticulata* could be associated with reinforcement of

xylem cell walls, since expression of auxin-related genes associated with cell-wall modification was up-regulated in the initial stage of infection. Curiously, this kind of resistance response is similar to that occurring with necrotrophic pathogens in the early stages of infection, where PAMPs, mediated by cell-wall degradation, can be recognized by the plant host, triggering an immunity response. Even though *X. fastidiosa* is not a necrotrophic organism, this bacterium can degrade plant cell walls in xylem vessels (Pérez-Donoso *et al.*, 2010) and be recognized by the plant host through some as yet unknown mechanism. Consistent with this hypothesis, Niza *et al.* (2015) recently showed that *X. fastidiosa* remains trapped in primary xylem of resistant plants due to lignin accumulation that coincides with the initial stage of infection (Fig. 3). In susceptible hosts, the bacterial strains were able to colonize primary and secondary xylem vessels, with lignification of primary xylem cells delayed and occurring later after infection, with no impairment of bacterial spread in the plant. Thus, the induction of lignification is suggested to be a physical defence response to *X. fastidiosa* infection in resistant plants, preventing the movement of this bacterium in the plant.

Citrus–Xanthomonas *interactions*

Xanthomonas is a genus of phytopathogenic Gram-negative bacteria that are known to cause disease in more than 200 plant families, including many economically important crops, such as rice, tomato, and citrus. *Xanthomonas citri* is the etiological agent of citrus canker, one of the most devastating diseases that affects citrus orchards worldwide. Initially, *X. citri* grows on leaf surfaces in structured biofilms (Rigano *et al.*, 2007) and it then enters the plant through stomata or injuries, and colonizes the mesophyll parenchyma.

Citrus canker infection is characterized by raised water-soaked lesions, which further progress to form the cankers. When trees are severely affected, infections cause premature fruit drop and defoliation, resulting in significant yield losses (Brunings and Gabriel, 2003). To date, there is no cure for citrus canker, and preventive copper spray application is one of the unique control measures that can be adopted, other than plant eradication (Behlau *et al.*, 2014).

There are three main types of citrus canker described, based on the strains that cause disease and the aggressiveness of the symptoms. Type A is the most aggressive cancrosis and is caused by *X. citri* (syn. *X. axonopodis* pv. *citri*). It originated in Asia and is able to infect all citrus varieties without any true resistance being recognized, despite the field tolerance described for some citrus genotypes such as 'Muscia' (*C. reticulata*) (Brunings and Gabriel, 2003; Carvalho *et al.*, 2015). Two other variants of Type A citrus canker are known: A*, which was first described in southern Asia in 1998, and Aw (Wellington strain), which was isolated in Florida in 2003. Both Aw and A* have a narrow host range, infecting only Mexican lime (*C. aurantifolia*) and alemow (*C. macrophylla*) (Vernière *et al.*, 1998; Sun *et al.*, 2004). Type B is also known as 'false canker' and was first described in Argentina in 1923 but has also been detected in Uruguay and Paraguay. It is virtually restricted to *C. limon*, although it can cause mild infections in all citrus varieties. Type C was isolated in São Paulo (Brazil) and, like Aw and A* strains, it is restricted to *C. aurantifolia* (Moreira *et al.*, 2010). Cancrosis of both Types B and C are caused by *Xanthomonas fuscans* subsp. *aurantifolli* strains.

Lipopolysaccharide (LPS) and adhesin are virulence factors that can be recognized as PAMPs. LPS has a role in the activation of basal defences in both host (*C. sinensis* 'Valencia late') and non-host (*Nicotiana tabacum* 'Petit Havana') plants (Petrocelli *et al.*, 2012). Similarly, the adhesin XacFhaB was also involved in the triggering of plant defence responses. The

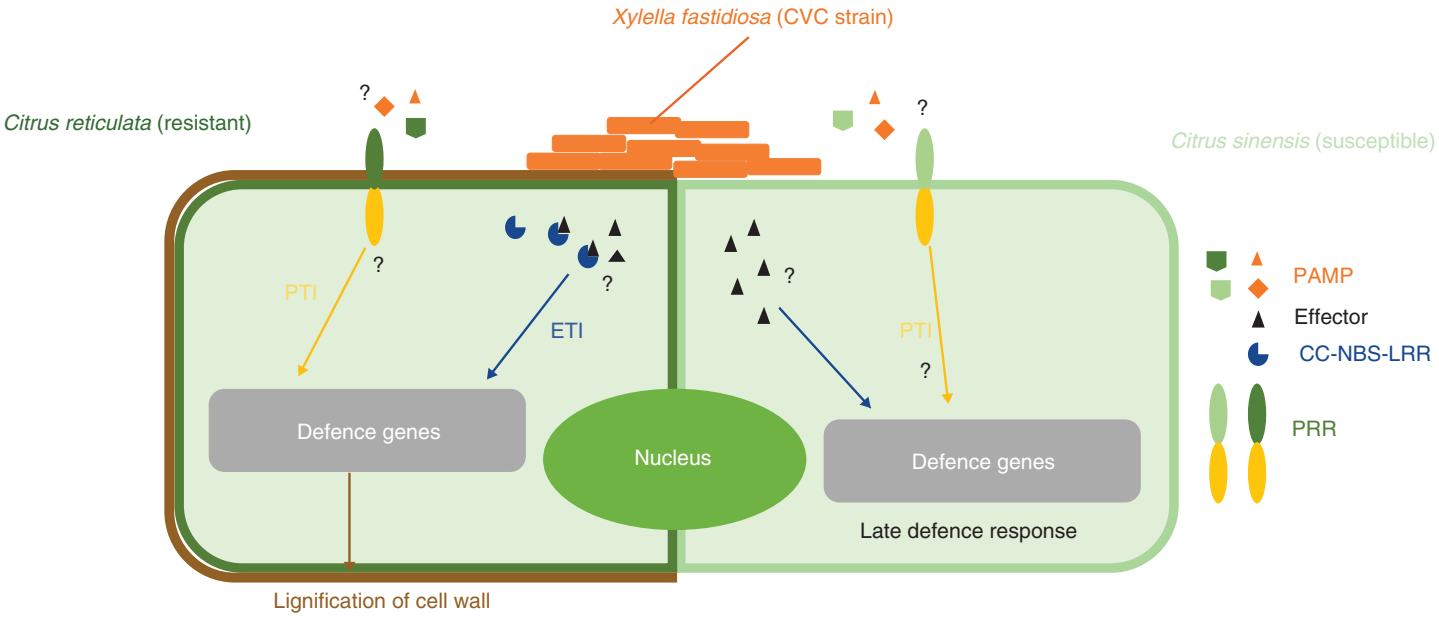

FIG. 3. Schematic representation of *X. fastidiosa* interaction with resistant and susceptible genotypes.

role of the three XacFhaB regions as PAMPs was investigated and all adhesin regions were able to induce basal immune responses in host and non-host plants. When citrus leaves were pre-infiltrated with XacFhaB regions, an inhibition of *X. citri* growth was observed, confirming the induction of defence responses and control of citrus canker (Garavaglia *et al*., 2016).

Another molecule recognized as a PAMP is flagellin. Studies using *flg22* of *X. citri* (*flg22Xcc*) 306 have shown that, in susceptible citrus genotypes 'Duncan' grapefruit and 'Navel' orange, no significant ROS production or PTI/ETI was associated with flg22*Xcc* treatment. However, resistant genotypes 'Nagami' kumquat and 'Sun Chu Sha' showed higher ROS production following *flg22Xcc* treatment (Shi *et al*., 2015), indicating that these plants are able to recognize this PAMP (Fig. 4).

Most *Xanthomonas* genomes sequenced to date have the *hrp/hrc* gene cluster, which render these cells capable of assembling a fully functional T3SS (Ryan *et al*., 2011). The needle-like structure formed by the T3SS proteins is responsible for the delivery of effectors directly inside the host cell (Fig. 4). All citrus canker-causing strains have similar T3SS gene clusters (Moreira *et al*., 2010; Neha Jalan *et al*., 2013) but there are differences among their effector pool (Moreira *et al*., 2010). The presence of the T3SS is necessary for full virulence of *X. citri*, which has been demonstrated in many studies (e.g. Laia *et al*., 2009). Therefore, the T3SS secreted effectors will be presented in more detail below.

Comparative analysis of the genomic sequence of four citrus canker-causing *Xanthomonas* [*X. citri* 306 (da Silva *et al*., 2002) *X. citri* Aw (Jalan *et al*., 2013) and *X. fuscans* B and C (Moreira *et al*., 2010)] reveals that they have 19 common effectors, which are therefore considered as the 'core' citrus canker effector pool. Among these, seven are also found in all other *Xanthomonas* genomes (avrBs2, xopK, xopL, xopQ, xopR, xopX and xopZ), and are considered the core effector set for this genus. The other 12 genes (xopA, xopE1, xopE3, pthA4 and/or its functional homologues, xopI, xopV, xopAD, xopAI, xopAK, xopAP, hpaA and hrpW) were found in the four citrus canker strains mentioned above (Neha Jalan *et al*., 2013).

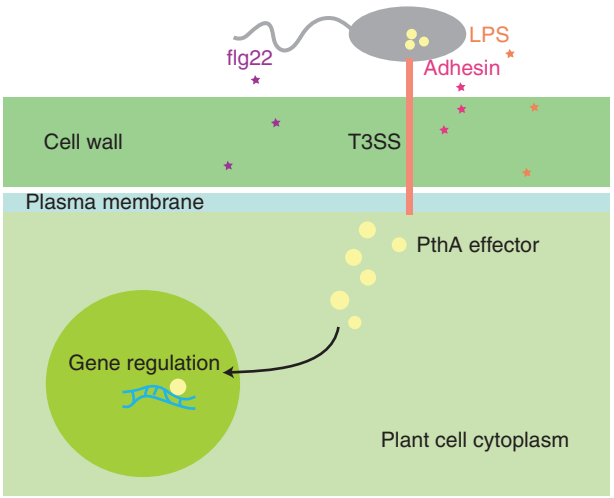

FIG. 4. PAMPs and effectors in *X. citri*. The known PAMPs for *X. citri* flagellin (flg22), adhesion and lipopolysaccharides (LPS) are present and enter the host cell. The T3SS-delivered effectors, such as PthA4 and its homologues, are injected into the host cell and travel to the nucleus, where they can act as transcriptional regulators.

The genes *xopAF* and *avrGf1* (=*xopAG*) are completely absent only in *X. citri* 306 (Neha Jalan *et al*., 2013). XopAF seems to promote the growth of *X. citri* Aw in Mexican lime (*C. aurantifolia*), contributing to virulence, while the presence of the AvrGf1 effector (= XopAG) is responsible for the HR observed in grapefruit (*C. paradisi*), restricting the host range of *X. citri* Aw (Rybak *et al*., 2009; Escalon *et al*., 2013; Jalan *et al*., 2013). Deletion of *avrGf1* enabled *X. citri* Aw to colonize grapefruit even though the symptoms were less severe than those caused by *X. citri* 306 (Rybak *et al*., 2009). In contrast, for sweet orange, symptoms were not visible in deletion mutants infecting this cultivar, suggesting that other factors may be acting in this host range restriction (Neha Jalan *et al*., 2013). A similar effector (AvrGf2) is found in *X. fuscans* C, also inducing ETI in grapefruit (Gochez *et al*., 2015). Interestingly, *X. fuscans* B has an almost identical gene coding for *avrGf2*, but its sequence is interrupted by a transposon, indicating that its ability to infect grapefruit may be due to the absence of a fully functional protein (Moreira *et al*., 2010).

Besides XopAG, Moreira *et al*. (2010) found other gene-coding effectors that occur in *X. fuscans* strains but are absent in the *X. citri* 306 genome, such as *xopB*, x*opE4*, *xopJ* (*avrXccB*) and x*opAF* (*avrXv3*). The gene sequence of *xopE4* is similar to *avrXopE3*, but due to its low amino acid sequence identity (31 %), it was considered a different effector. Another effector gene (*avrXccA2*) was found only in a few strains of *X. fuscans*, but it was not found in the two sequenced strains (*X. fuscans* B and C). In addition, *X. fuscans* C is uniquely depleted in effectors such as *xopE2*, *xopN*, *xopP*, *xopAE* (Moreira *et al*., 2010) and XopAQ (Neha Jalan *et al*., 2013). A general overview of the T3SS-delivered effectors is shown in Fig. 5.

One of the most important T3SS-delivered effectors found among the citrus canker strains is PthA4 and its homologues. This protein is a T3SS effector delivered inside the plant cell and is a key effector responsible for canker development. Its presence alone is capable of inducing canker formation, while its absence suppresses the appearance of cankers (Al-Saadi *et al*., 2007). PthA and its homologues are members of the transcription activator-like (TAL) effector family of proteins (formerly known as the AvrBs3/PthA family).

The TAL effectors are proteins that can control gene expression of the host cell they are delivered into, where they can enter the cell nucleus and act as transcriptional regulators favouring pathogen development. Therefore, TAL effectors must target specific DNA sequences which can be accomplished due to the presence of conserved (almost identical) repeats that include a repeat variable di-residue (RVD). Each of the RVDs recognize one nucleotide and the juxtaposed RVDs target a given DNA sequence (Streubel *et al*., 2012).

Xanthomonas citri 306 has four nearly identical copies of the TAL-effector PthA4, all present on plasmids, but only one of them, PthA4, is the main effector involved in canker formation (Swarup *et al*., 1992). This effector does not determine host range in citrus species and varieties, but does restrict this pathogen to citrus. It is recognized by non-host plants, triggering ETI in all non-citrus plants tested to date (Swarup *et al*., 1992).

PthA homologues are known to elicit the classical symptoms of citrus canker, which are hyperplastic and hypertrophic

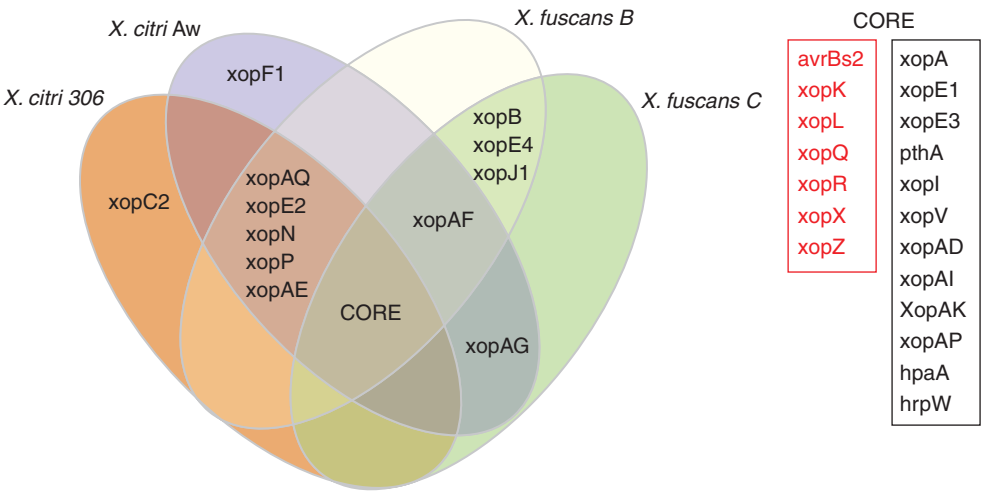

FIG. 5. General overview of T3SS-delivered effectors found in citrus-canker causing *Xanthomonas* species. The centre of the Venn diagram represents the core effectors found among these genomes (see box on the right), with these *Xanthomonas* core effectors shown in red.

water-soaked lesions that become thicker and darker, ultimately causing the epidermis to rupture, spreading the bacteria (Brunings and Gabriel, 2003). Recently, among other possibilities, one of the targets of PthA4 was found to be the plant gene CsLOB1 (a member of the lateral organ boundaries family of transcription factors), which forms pustules when over expressed. Interestingly, the activation of this gene was observed with all the PthA4 variants found in citrus canker-causing *Xanthomonas* (Li *et al*., 2014). However, not only CsLOB seems to be controlled by PthA effectors, and the regulation of many genes is possibly required, with the dioxygenase gene (DIOX) being another possible candidate (Abe and Benedetti, 2016). Although PthA4 is indeed the eliciting factor needed for canker formation, the other PthA copies present in *X. citri* 306 seem to have an important role in canker development, especially in some citrus varieties (Abe and Benedetti, 2016).

Citrus–*HLB interaction*

HLB is a century-old disease that has emerged as the most destructive citrus disease worldwide in the past few decades (Wang and Trivedi, 2013). HLB is associated with three phloem-limited Gram-negative bacteria belonging to the genus *Liberibacter* (Bové, 2014). No efficient method for cultivation has been found to isolate these bacteria to date, and thus they remain in the '*Candidatus*' status (Fleites *et al*., 2014). HLB-associated '*Candidatus* Liberibacter' are named according to the place they were first detected: '*Candidatus* Liberibacter asiaticus' (*Ca*Las – Asia), '*Candidatus* Liberibacter africanus' (*Ca*Laf – Africa) and '*Candidatus* Liberibacter americanus' (*Ca*Lam – America) (Garnier *et al*., 2000; Wang and Trivedi, 2013). Owing to its wide distribution, '*Ca*Las' has been the most studied bacteria of the genus '*Ca*. Liberibacter' related to citrus HLB. *Diaphorina citri* and *Tryoza eritrea* are the main insect vectors of the bacteria associated with HLB disease; the first one is responsible for transmission of 'CaLas' and 'CaLam' in Asia and America, and *T. eritrea* transmits 'CaLaf' in Africa (Bové, 2014).

Due to its negative effects on citrus yield, the disease is studied using different approaches with the aim of developing HLB-resistant citrus varieties. Several genome sequences were obtained from isolates of *Liberibacter* species (da Graça *et al*., 2016). These sequences have been extensively explored and have led to new studies on the biology of the pathogen.

Thus far, no conclusive pathogenicity mechanism of the '*Ca*. Liberibacter spp.' has been identified (da Graça *et al*., 2016). Phloem dysfunction appears to be the primary alteration that might determine the emergence of other symptoms (Koh *et al*., 2012). When studying this alteration, Zou *et al*. (2012) showed that '*Ca*Las' encodes a flagellin and a hook-associated protein (fla) with PAMP activity. In this research, a synthetic $flg22_{Las}$ peptide induced callose deposition in *N. benthamiana*, although with a weaker response than observed in other well-characterized plant–pathogen systems. In microscopic analysis of '*Ca*Las'-infected citrus, accumulation of callose was observed in sieve plates (Koh *et al*., 2012). Excessive callose deposition in phloem plasmodesmata may interrupt photoassimilate distribution along the source and sink system, causing starch over-accumulation in leaf chloroplasts (Koh *et al*., 2012). Other factors such as phloem protein (PP2) accumulation in sieve plates and collapse of phloem cells might contribute to phloem dysfunction (J.-S. Kim *et al*., 2009) (Fig. 6).

Besides anatomical alterations, several metabolic imbalances and genetic reprogramming are observed in HLB-infected plants (Aritua *et al*., 2013; Mafra *et al*., 2013; Chin *et al*., 2014). A protein with potential salicylate hydroxylase activity was identified in the '*Ca*Las' genome, which might convert salicylic acid into catechol (Wang and Trivedi, 2013). Salicylic acid is a hormone that plays an important role in induction of plant defence systems against biotrophic pathogens (Yusuf *et al*., 2013). Therefore, '*Ca*Las' might evade plant defences by modulating overall defence of its hosts. This hypothesis is supported by studies demonstrating depression of the salicylic acid pathway in susceptible citrus plants (Xu *et al*., 2015) (Fig. 6).

Another interesting characteristic found in '*Ca*. Liberibacter spp.' is the presence of prophages integrated in their genomes (Zhang *et al*., 2011). Many bacterial pathogens contain

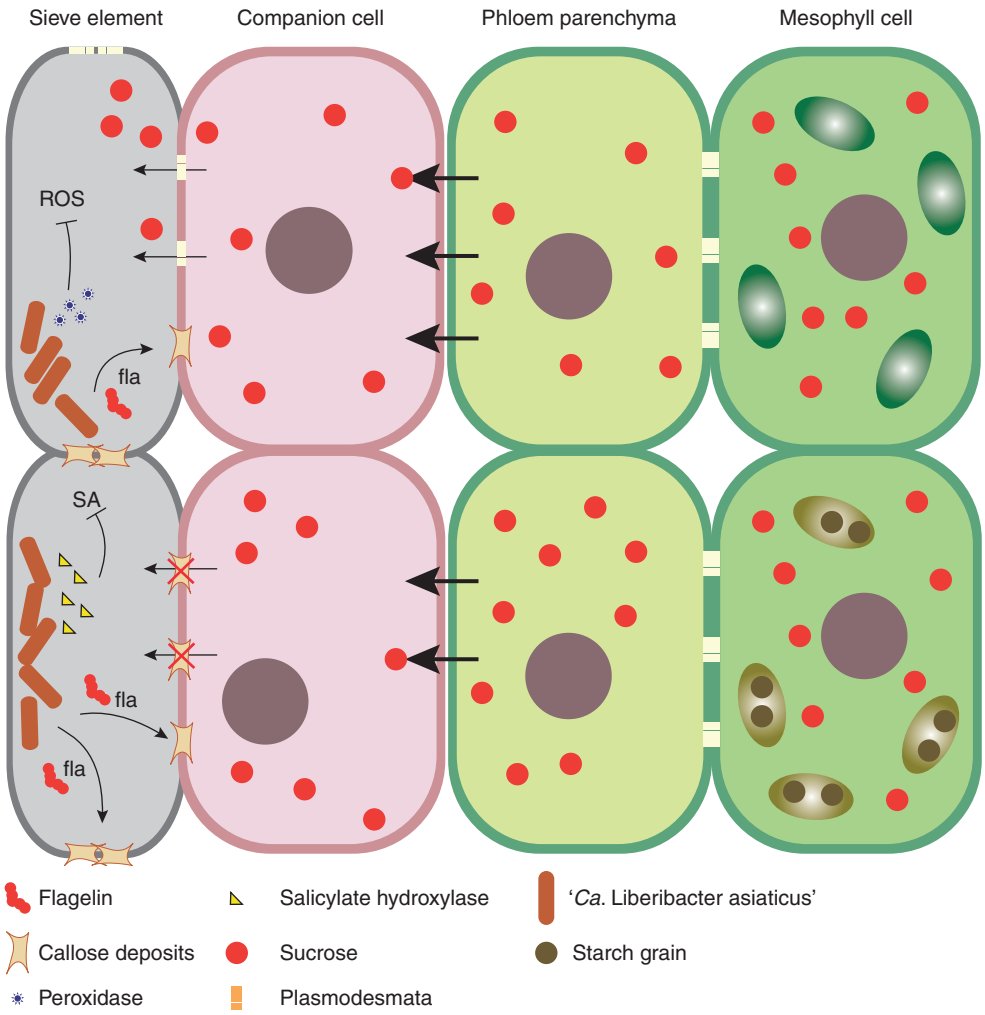

FIG. 6. An interaction model between citrus and '*Candidatus* Liberibacter asiaticus'. Reported components of the liberibacter possibly associated with its virulence mechanisms regarding suppression of host immunity and manipulation of its physiology. Flagellin components induce blockage of phloem plasmodesmata and impair sap flow between cells. Starch grains accumulate in chloroplasts in response to several changes in enzymatic activities. Salicylic acid might be broken down into catechol by hydroxylases and reactive oxygen species are suppressed by the activity of peroxidases of the bacteria.

prophages or phage remnants integrated in their genomes that encode virulence factors (Menouni *et al*., 2014). '*Ca*Las', for example, carries two predicted prophages: an excision prophage (SC2) and a chromosomally integrated prophage (SC1) (Fleites *et al*., 2014). SC1 replicates and forms phage particles in the phloem of '*Ca*Las'-infected periwinkle and in citrus, but not in the psyllids (Fu *et al*., 2014). Both phages encode two proteins with peroxidase activity (Zhang *et al*., 2011). These peroxidases might protect the bacteria against ROS produced by the plant during infection (Jain *et al*., 2015). The SC1 prophage also encodes functional holin (SC1_gp110) and endolysin (SC1_gp035) proteins that might be implicated in bacterial membrane lysis and cell-wall degradation during bacteriophage egress (Fleites *et al*., 2014). An incomplete phage/prophage variant (iFP3) derived from recombination of FP1 (SC1) and FP2 (SC2) was reported in '*Ca*Las' (Zhou *et al*., 2013). iFP3 is absent or detected at low levels in psyllids but it is abundant in host plants. Furthermore, it might be associated with blotchy mottle symptoms and disease development (Zhou *et al*., 2013; Pitino *et al*., 2014).

Given that '*Ca*. Liberibacter spp.' are intracellular pathogens, there is a conjecture that the bacteria secrete effector proteins directly into host cell cytoplasm and modulate its physiology (Puttamuk *et al*., 2014). '*Ca*. Liberibacter spp.' contain a general secretory pathway, which might contribute to the secretion of these molecules (Hao *et al*., 2013). A few studies have reported findings about potential effectors of '*Ca*. Liberibacter spp.'. A putative serralysin was predicted in the '*Ca*Las' genome (Cong *et al*., 2012). This protein is a metalloprotease associated with the type I secretion system (T1SS) and might act as a virulence factor. It is very likely that serralysin plays an important role in infection as an anti-immune mechanism by degrading host proteins, as already described in other pathosystems (Felfoldi *et al*., 2009). A protein containing a von Willebrand factor type A domain (vWA) was also identified in the '*Ca*Las' genome (Cong *et al*., 2012). This protein is

predicted to be associated with cell adhesion, migration and signal transduction, although it has yet to be well characterized in '*Ca*Las'. Other candidates for virulence factors are reported in the analyses carried out by Cong *et al*. (2012).

A putative effector (CLIBASIA_05315) was identified in the '*Ca*Las' genome (Pitino *et al*., 2016). This protein fused to GFP was localized in chloroplasts of *Nicotiana benthamiana* after transient expression in leaf tissue and induced cell death associated with H_2O_2 accumulation, electrolyte leakage and callose deposition. This protein was also localized in chloroplasts of transgenic citrus and resulted in leaf chlorosis and plant growth retardation. Another potential effector candidate named LasAI was also identified, which induced an increase in the number of root hairs when expressed in *A. thaliana* and an increased number of trichomes when transiently expressed in *N. benthamiana* (Pitino *et al*., 2015).

Despite the recent advances in identification of effectors, a model that explains the pathogenicity mechanism of the bacteria associated with HLB has not been established. Furthermore, the group of liberibacters might behave as an obligate 'energy parasite' rather than a pathogen (Haapalainen, 2014). Several research groups are currently focusing on the identification and characterization of effector proteins of '*Ca*. Liberibacter spp.', and it is expected that in a few years we will have an improved view of this pathosystem evolution (Table 2).

CITRUS–FUNGI INTERACTIONS

Citrus post-bloom fruit drop and key lime anthracnose

The genus *Colletotrichum* comprises at least 600 species and includes a number of important pathogens that cause economically significant losses on various crops worldwide. Disease caused by this group of fungi is known as anthracnose (O'Connell *et al*., 2012). *Colletotrichum* species are characterized by a distinctive hemibiotrophic lifestyle (O'Connell *et al*., 2012). Infection occurs through a brief biotrophic phase followed by a necrotrophic phase, in which secondary hyphae spread throughout the host tissue. Production of orange–brown lesions on petals of open citrus flowers, induction of abscission of young fruit and formation of persistent calyces are characteristic symptoms of post-bloom fruit drop (PFD) (de Goes *et al*., 2008). In key lime, *C. acutatum* infects all parts of the plant, causing anthracnose symptoms associated with key lime anthracnose (KLA) (You and Chung, 2007).

Damm *et al*., (2012) described species of the *C. acutatum* complex and reported that citrus is attacked by more than one species of this complex. Pinho and Pereira (2015) identified a new species, *C. abscissum*, as being the agent responsible for PFD (Crous *et al*., 2015). *Colletotrichum gloeosporioides* was reported as the casual agent of PDF, although it exhibits lower pathogenicity levels in comparison with the *C. acutatum* complex (Lima *et al*., 2011).

The high variability and ability of this fungus to infect all sweet orange varieties and key lime must be related to a large arsenal of effector proteins in its genome. Similarly, in the genomes and transcriptomes of *C. higginsianum* infecting *A. thaliana* and *C. graminicola* infecting maize, 365 and 177 candidate secreted effectors were found, respectively (O'Connell *et al*., 2012).

Currently, studies focusing on understanding plant–pathogen interactions have shown that activation of these effectors is orchestrated and in some cases it follows specific patterns, such as expression in waves during infection (Giraldo and Valent, 2013). In hemibiotrophic fungi, gene expression studies have increasingly shown highly controlled gene regulation specific for each infection phase (Giraldo and Valent, 2013).

Colletotrichum spp. proteins related to pathogenicity have not yet been fully characterized and the identification of effector proteins in citrus is underway. The restriction enzyme-mediated integration (REMI) technique, which was used by Chen *et al*. (2005) for transformation of *C. acutatum*, resulted in six mutants not pathogenic in key lime. The *KLAP1* gene was responsible for the loss of *C. acutatum* pathogenicity (Table 3). *KLAP1*-null mutants were unable to develop the penetration stage on leaves of key lime, but were able to cause orange–brown lesions similar to the wild-type on flower petals. The actual function of *KLAP1* remains uncertain but this gene

TABLE 2. *PAMPs and putative effectors of* 'Ca. *Liberibacter asiaticus*'

	Gene	Description	Reference
PAMPs	Flagellin	flagellin	Zou *et al*. (2012)
	Peroxidase	hypothetical protein SC2_gp095	Jain *et al*. (2015)
	Las5315	hypothetical protein	Pitino *et al*. (2016)
	LasAI	hypothetical protein	Pitino *et al*. (2015)
Putative effectors	Protein serine/tyrosine phosphatase	hypothetical protein	Cong *et al*. (2012)
	Serralysin	Serralysin	Wang and Trivedi (2013)
	Haemolysin	Haemolysin	
	Salicylate hydroxylase	Monooxygenase FAD-binding protein	

TABLE 3. *Candidate effector genes identified in* Colletotrichum acutatum *of citrus pathogen*

	Gene	Description	Reference
Putative effectors	*KLAP1*	Hypothetical transcription activator	Chen *et al*. (2005)
	$PacC^{KLAP2}$	Hypothetical PH regulation	You *et al*. (2007)

may encode a putative transcription activator necessary for penetration of the hyphae on key lime leaves, suggesting that these proteins may be effectors.

Unlike KLAP1, mutants of the $PacC^{KLAP2}$ gene characterized by You *et al*. (2007) were less effective in causing anthracnose in key lime and sweet oranges, demonstrating that $PacC^{KLAP2}$ is a common gene for virulence of *C. acutatum* in both pathosystems (Table 3). *Colletotrichum acutatum* transformants not expressing transcripts of the $PacC^{KLAP2}$ gene were unable to grow at high pH. However, they are capable of forming appressoria on the host leaves and flowers, but fail to colonize the surrounding tissue. Enzymatic tests showed a decrease in alkaline phosphatase activity, proteases and cellulases in $PacC^{KLAP2}$ null mutants (You *et al*., 2007). Absence of these enzymes prevents fungal development at high pH and should be required for *C. acutatum* pathogenesis. CUT1 gene transcripts decreased indicating that $PacC^{KLAP2}$ also regulates the expression of cutinases (You and Chung, 2007). If the gene expression pattern of *C. higginsianum* were similar to *C. acutatum* when infecting citrus species, this would suggest that the protein coded by the $PacC^{KLAP2}$ gene is a 'candidate effector'. This protein is secreted by the appressorium and it seems to be important for the development of pathogenicity in the host tissue.

Alternaria brown spot in citrus

Alternaria brown spot, caused by *Alternaria alternata*, is an important disease of tangerines and their hybrids, affecting leaves, twigs and immature fruit (Canihos *et al*., 1999). This fungus is also responsible for rough lemon brown spot (Timmer *et al*., 2003). There are two different pathotypes that produce host-specific toxins (HSTs), which are responsible for causing the disease (Tsuge *et al*., 2013).

One of the main barriers to prevent the fungal penetration of plant pathogens is the cell wall, and therefore many fungi secrete extracellular enzymes that can degrade cell-wall polymers. One of these enzymes produced by the genus *Alternaria* is endopolygalacturonase (endoPG) (Isshiki *et al*., 1997). An *A. citri* mutant for this gene resulted in a significant reduction in its ability to cause black rot symptoms in citrus (Isshiki *et al*., 2001). There is no *Alternaria* endoPG receptor described in *Citrus*; however, in *A. thaliana*, a receptor was identified as a leucine-rich repeat receptor-like protein (RESPONSIVENESS TO BOTRYTIS POLYGALACTURONASES1, RBPG1), which recognizes fungal endoPGs (Zhang *et al*., 2014).

The laccase enzyme was suggested as an elicitor of the genus *Alternaria* and could be involved in the pathogenesis of *A. alternata* in *Citrus*. After challenging 'Fortune' mandarin, *C. limon* and *C. paradise* with *Alternaria*, the flavonoid degradation pathway was activated in the host plants in association with the *de novo* synthesis of the phytoalexin scoparone. This metabolism of flavonoids is caused by an extracellular laccase, which utilizes *Citrus* flavonoids as a substrate (Díaz *et al*., 2015). Another elicitor from *Alternaria* is β-1,3-,1,6-oligoglucan, which is a fungal cell-wall component. This glucan, when applied to a tobacco plant model (BY-2), induced chitinase activity (Shinya *et al*., 2006). In soybean, the PRR recognizing *Phytophthora megasperma* β-glucan was identified as the β-glucan binding protein (GBP) (Umemoto *et al*., 1997). However, this MAMP and its corresponding PRR have not been studied in citrus species or in any other organisms.

Effectors of necrotrophic pathogens include phytotoxins and proteinaceous effectors (Wang *et al*., 2014). Phytotoxins can be divided into general toxins (non-HSTs), to which many plant species are sensitive, and HSTs, where sensitivity is restricted to specific host genotypes (Oliver and Solomon, 2010). Based on their chemical structure, phytotoxins are classified as polyketides, non-ribosomal peptides, alkaloids, terpenes or metabolites of mixed biosynthetic origin (Stergiopoulos *et al*., 2012).

Recent studies have shown that several necrotrophs secrete HSTs, which play a crucial role in disease outcome (Izumi *et al*., 2012). Therefore, the toxins may be considered as the major group of effectors of necrotrophic fungi, since they have characteristics of avirulence genes (Stergiopoulos *et al*., 2012). These toxins secreted by necrotrophic fungi are detected by R-genes of susceptible plants in order to trigger the HR and initiate cell death. This phenomenon is known as dominant susceptibility (Liu *et al*., 2009). Thus, interactions between necrotrophic effectors and genes for susceptibility of plants are called inverse interactions of the gene-for-gene theory (Oliver and Solomon, 2010).

In *Citrus*, the two pathotypes of *A. alternata* are identified according to the production of HSTs. One is the tangerine pathotype, which produces ACT-toxin specific to tangerine (*C. reticulata* 'Blanco') and their hybrids. The other is the rough lemon pathotype, which affects rough lemon (*C. jambhiri* 'Lush') and Rangpur lemon (*C. limonia* 'Osbeck'), and produces ACR-toxin (Tsuge *et al*., 2013). These HSTs are essential for host-selective infection and disease development (Tsuge *et al*., 2013).

Due to the lifestyle of *Alternaria*, cell death is the main mechanism for success of the pathogen. HSTs produced by this species have a central role in the host cell-death induction mechanism and are critical for successful pathogenesis. Other classic effectors, which evade recognition or hinder the defence, do not necessarily cause disease, given that the toxins are always identified as the central component in pathogenesis.

The chemical structure of the ACT-toxin consist of three parts: 9,10-epoxy-8-hydroxy-9-methyl-decatrienoic acid (EDA), valine and a polyketide (Kohmoto, 1993). One putative target site of ACT-toxins in tangerine is the plasma membrane (Kohmoto, 1993). These toxins are quickly translocated through the vascular system, causing rapid electrolyte leakage and necrotic lesions along the veins (Chung, 2012). After infection of citrus leaves by *A. alternata*, the induction of fast lipid peroxidation and accumulation of hydrogen peroxide (H_2O_2) occur (Lin *et al*., 2011) (Fig. 7).

The rough lemon pathotype of *A. alternata* produces a host-selective ACR-toxin and causes Alternaria leaf spot disease of rootstock species such as rough lemon (*C. jambhiri*), Rangpur lime (*C. limonia*) and a hybrid of rough lemon and acid mandarin, Rangpur lime (*C. limonia* 'Osbeck') (Akimitsu *et al*., 2003). The structure of ACR-toxin I consists of a polyketide with an α-dihydropyrone ring in a 19-carbon polyalcohol (Akimitsu *et al*., 2003). ACR-toxins are polyketide secondary metabolites and the ACRTS2 gene that encodes a polyketide synthase (PKS) essential for biosynthesis of these toxins. This gene was identified in the rough lemon pathotype (Izumi *et al*.,

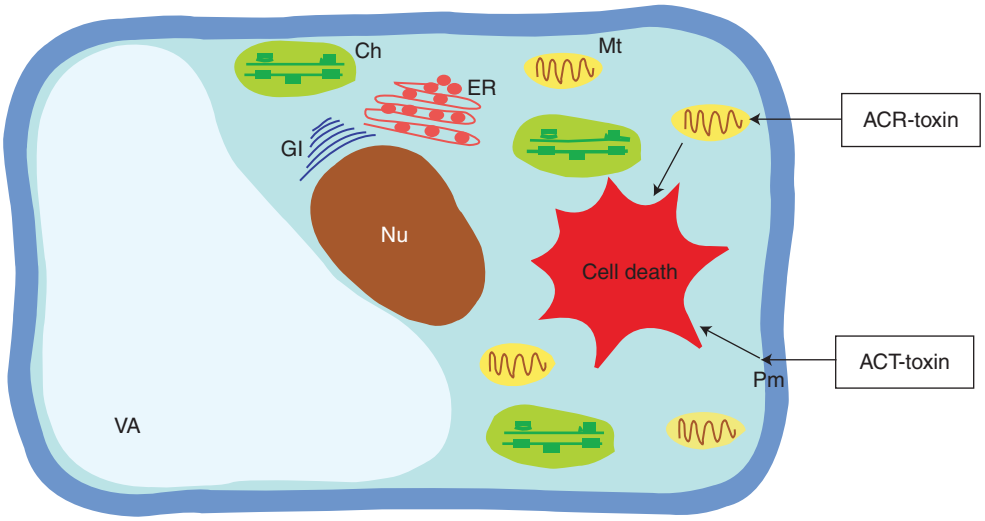

FIG. 7. Schematic representation of target sites of toxins produced by *A. alternata*. The putative target site of ACT-toxins in tangerine is the plasma membrane. After the infection of citrus leaves, induction of fast lipid peroxidation and accumulation of hydrogen peroxide (H_2O_2) occurs, resulting in cell death. The target of ACR-toxin is the mitochondrion, which after contact with the host plant causes a rapid increase in electrolyte leakage and consequent cell death. Ch, chloroplast; ER, endoplasmic reticulum; Gl, Golgi apparatus; Mt, mitochondrion; Nu, nucleus; Pm, plasma membrane; Va, vacuole.

TABLE 4. *The effectors involved in the pathogenesis of* A. alternata *in* Citrus

	Gene	Description	Reference
PAMP/DAMP	Endopolygalacturonase (endoPG)	enzyme	Isshiki *et al.* (1997)
	laccase enzyme	enzyme	Díaz *et al.* (2015)
	β-1,3-, 1,6-oligoglucans	glucan	Shinya *et al.* (2006, 2007)
Putative effectors	ACT-toxin	toxin	Kohmoto (1993)
	ACR-toxin	toxin	Akimitsu *et al.* (2003)

2012). The target of ACR-toxin is the mitochondrion (Kohmoto *et al.*, 1984). After coming into contact with the host plant, ACR-toxins cause water congestion and veinal necrosis, and induce a rapid increase of electrolyte leakage (Kohmoto, 1979) (Fig. 7).

The effectors, PAMPs, DAMPs as well as toxins involved in the pathogenesis of *A. alternata* in *Citrus* are shown in Table 4.

The involvement of chitinases (Ch) and β-1,3-glucanases (Glu) in the defence response against *A. alternata* was investigated in *C. limon* seedlings. Following inoculation of the fungus, increased activity of these enzymes was observed with the detection of a new Ch isoenzyme and of three new Glu enzymes (Fanta *et al.*, 2003).

Enhancement of the citrus plant immune system was observed after treatment of 'Fortune' mandarin with hexanoic acid (Hx). The diameter of the lesions was significantly reduced in plants treated with Hx and challenged with *A. alternata* compared with non-treated plants. Furthermore, treated plants showed an increase in callose deposition and activation of the jasmonic acid pathway (Llorens *et al.*, 2013).

Other defence mechanisms were reported in citrus plants exposed to toxins that do not cause disease. After inoculation of rough lemon with the tangerine pathotype, which produces an ACT-toxin that is not toxic to this plant, the induction of several defence-related genes such as chitinases (Gomi *et al.*, 2002*a*), lipoxygenase (Gomi *et al.*, 2002*b*), epoxide hydrolase (Gomi *et al.*, 2003*b*), hydroperoxide lyase (Gomi *et al.*, 2003*a*), chalcone synthase (Gotoh *et al.*, 2002), miraculin-like protein (Tsukuda *et al.*, 2006) and thaumatin-like protein (B.-G. Kim *et al.*, 2009) was observed.

CITRUS–OOMYCETES INTERACTIONS

Commonly mistaken as fungi because of morphological similarities, the oomycetes are a group of eukaryotic micro-organisms that include pathogens of insects, crustaceans, fish, vertebrates, micro-organisms and plants. Modern molecular phylogenies based on rRNA sequences, amino acid data for mitochondrial proteins and four protein-encoding chromosomal genes have been used to identify the oomycetes as a unique lineage of stramenopile eukaryotes, unrelated to true fungi but closely related to heterokont photosynthetic algae (ADL *et al.*, 2005; Kamoun *et al.*, 2015).

The genus *Phytophthora* consists of more than 100 plant-pathogenic species with worldwide distribution. As pathogens they infect more than 250 plant families and damaging crops and natural ecosystems (Kroon *et al.*, 2012). Several *Phytophthora* species have been associated with disease in citrus plants. The predominant species in citrus orchards and nurseries worldwide include: *P. boehmeriae* Saw., *P. cactorum* (Lebert & Cohn) Srhöter, *P. capsici* Leonian, *P. cinnamomi* Rands, *P. citricola* Saw., *P. citrophthora* (Sm. & Sm.) Leonian,

P. drechsleri Tucker, *P. hibernalis* Carne, *P. megasperma* Drechsler, *P. palmivora* (Butler) Butler, *P. nicotiane* (=*P. parasitica*) Dastur., *P. parasitica* and *P. citrophthora* (Luz, 2001). *Phytophthora parasitica* is the main citrus pathogen due to its geographical distribution and severity (Panabieres *et al.*, 2016).

Phytophthora species are able to secrete two types of effectors related to their localization in plant tissues: the apoplastic or extracellular effectors, such as elicitins and NPP-like effectors; and cytoplasmic effectors, such RxRL and Crinkler effectors (CRNs), which possess special amino acid motifs in their structure enabling their entry inside cells independent of the presence of the pathogen (Fig. 8) (Hogenhout *et al.*, 2009; Kamoun, 2009).

Apoplastic Phytophthora *effectors*

Elicitins Elicitins are extracellular proteins with low molecular weight (about 10 kDa) which are secreted by most members of the genus *Phytophthora* (Oßwald *et al.*, 2014). The first characterized elicitin was INF-1 from *P. infestans*. INF-1 induces a strong HR in tobacco plants (Kamoun *et al.*, 1998). Sharing features of PAMPs, elicitins are widely used to induce HR and to study defence in plants. Studies on the crystallography and functional characterization of elicitins in model plants such as *A. thaliana* and *N. benthamiana* have been extensive. Usually, elicitins lead to local and systemic responses in plants after inducing an oxidative burst in cells through efflux of K^+ and Cl^- and influx of Ca^{2-} (Fellbrich *et al.*, 2002). This phenomenon is found not only in members of the family Solanacea but also in Brassicacea plants. However, little is known about the role of these proteins in compatible interactions (Bhattacharjee *et al.*, 2006; Svozilová *et al.*, 2009).

Among the oomycetes pathogens of citrus plants, species such as *P. parasitica*, *P. citrophthora*, *P. citricola*, *P. capsici*, *P. drechsleri*, *P. palmivora* and *P. megasperma* are highlighted since they have several types of elicitins and elicitin-like proteins organized as multigenes. *Phytophthora parasitica*, for instance, secretes the elicitin ParA1 which induces a very strong HR in tobacco (Kamoun, 1993). A simple search for elicitin proteins in public databanks such as NCBI yields more than 500 elicitin-related proteins, 19 of which are characterized as elicitins and 489 are hypothetical elicitins (Geer *et al.*, 2010). The EST sequencing project for *P. parasitica* led to the identification of ten different elicitin classes (Panabières *et al.*, 2005). Most abundantly expressed are the class 1 proteins of parasiticein and encoded by at least four genes (ParA1.1–ParA1.4) (Panabières *et al.*, 2005). Parasiticein genes from classes 5 and 6 (PAR5 and PAR6) have N-terminal sequence similarities with a phospholipase from *P. capsici*, suggesting an involvement of PAR5 and PAR6 in membrane remodelling (Nespoulous *et al.*, 1999). For the *P. parasitica*–citrus interaction, it was found that elicitins were up-regulated at the later stages of infection, indicating elicitins are correlated with the late necrosis in tissues of susceptible varieties of citrus (Boava *et al.*, 2011).

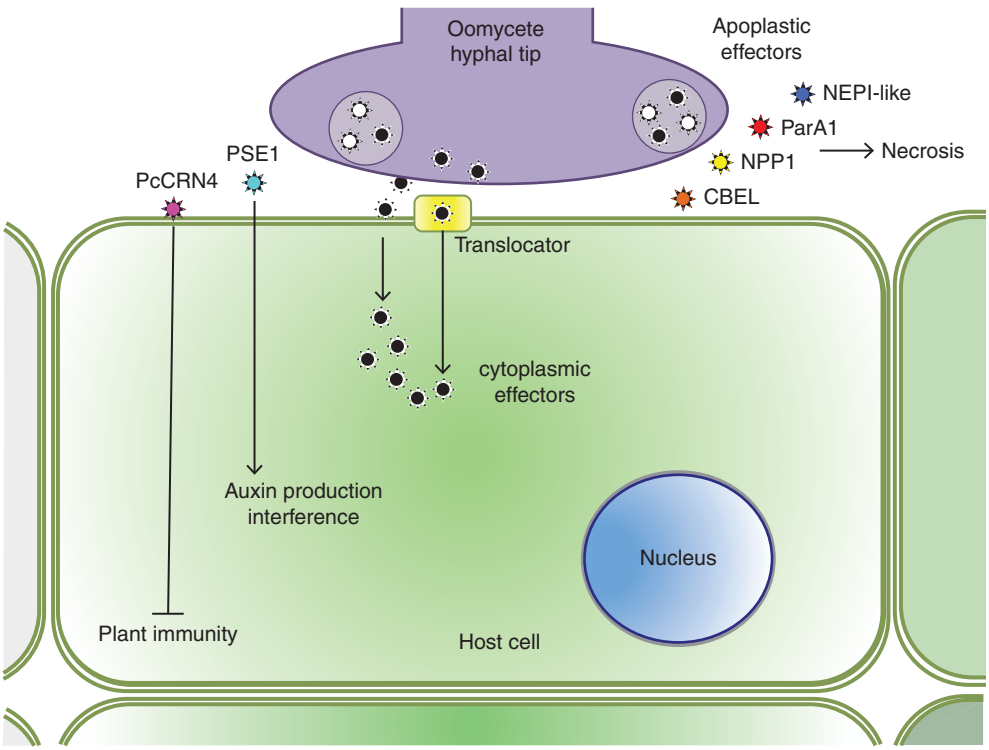

FIG. 8. Oomycete pathogens of citrus release apoplastic effectors such as NEP-like, ParA1, NPP1 and CBEL, which can elicit plant responses and necrosis and/or cytoplasmic effectors, such as PSE1 (RxLR effector) and PcCRN4 (Crinkler effector), which use the plant machinery (a translocator) to invade the cytoplasm and interfere with auxin production or suppress plant immunity, respectively.

Other Phytophthora *effectors*

Other reported effectors of *P. parasitica* that might have important roles in the interaction with citrus plants include: NEPl-like protein (necrosis and ethylene-inducing peptide), NPP1 (necrosis-inducing *Phytophthora* protein 1) (Fellbrich *et al.*, 2002), the gene family encoding apoplastic polygalacturonases (Yan and Liou, 2005; Wu *et al.*, 2008) and CBEL (cellulose-binding, elicitor and lectin activity) apoplastic effectors, which are purified from cell walls and, importantly, induce HR when infiltrated in leaves of tobacco and *A. thaliana* (Khatib *et al.*, 2004; Oßwald *et al.*, 2014). It is also suggested that the structure of the hyphal cell wall and attachment to cellulosic substrates, such as plant surfaces, depend on CBEL effectors (Gaulin *et al.*, 2002). The main role of the aforementioned effectors during infection of citrus plants by *P. parasitica* is still obscure.

Cytoplasmic Phytophthora *effectors*

RxLR effectors The proteins from the RxLR family are cytoplasmic modular effectors carrying a conserved amino acid motif in their N-terminal structure: RxLR (R: arginine; x: any amino acid; L: leucine; R: arginine) (Fig. 9) (Win *et al.*, 2007). The RxLR motif is particularly interesting because it enables the delivery of these proteins into the interior of cells using the plant machinery (Grouffaud *et al.*, 2008). One of the most studied RxLR effectors is the *P. infestans* AVR3a, which suppresses cell death induced by the elicitin INF-1 (Bos *et al.*, 2009).

RxLR effectors from *P. parasitica* were shown to be differentially expressed in the necrotrophic phase of infection of *A. thaliana* (Attard *et al.*, 2014). Through localization studies, GFP-labelled RxLRs were observed in hyphae and appressoria. Another study demonstrated that the *P. parasitica* RxLR effector PSE1 (penetrating specific effector 1) promotes infection of *A. thaliana* by interfering with auxin physiology (Evangelisti *et al.*, 2013).

Searches for RxLR effectors in the genome of *P. parasitica* INRA-310 (originally isolated from tobacco in Australia, but also pathogenic to citrus) at the Fungidb platform (fungidb.org) rendered 179 hits. An additional eight genomes of different *P. parasitica* isolates are now also available at the Broad Institute (olive.broadinstitute.org/projects/phytophthora_parasitica), each showing a repertoire of RxRL effectors. To our knowledge, no *P. parasitica* RxLR effector has yet been characterized functionally.

Crinkler effectors (CRN) Crinklers are cytoplasmic effectors originally described in *P. infestans*. Today, it is accepted that CRN effectors are secreted by most *Phytophthora* species as well as other plant-pathogenic micro-organisms such as *Hyaloperonospora arabidopsidis* (Baxter *et al.*, 2010), *Bremia lactucae* (Stassen and Van den Ackerveken, 2011) and *Pythium ultimum* (Lévesque *et al.*, 2010). The name Crinkler was originally used because of the crinkling phenotype of leaves infected with *P. infestans* (Torto *et al.*, 2003).

The structure of CRN effectors presents a highly conserved N-terminal amino acid domain: Leu-Xaa-Leu-Phe-Leu-Ala-Lys (LxLFLAK; Fig. 10) (Haas *et al.*, 2009). Functional characterization of CRN (PsCRN70) of *P. sojae* in *N. benthamiana* showed that the effector suppressed cell death induced by the INF-1 elicitin. INF-1 would act as a PAMP inducing cell death (Schornack *et al.*, 2010), while PsCRN70 would suppress the responses contributing to pathogen virulence (Rajput *et al.*, 2014).

The CRN family shows extensive expansion in all sequenced *Phytophthora* species, including *P. parasitica* (Tyler *et al.*, 2006; Haas *et al.*, 2009). *Phytophthora capsici* secretes PcCRN4, which is an effector essential to pathogen virulence, since it suppresses plant-immunity responses (Mafurah *et al.*, 2015). Searching for CRN effectors in the genome of *P. parasitica* INRA-310 at the Fungidb platform (fungidb.org) renders 26 hits. However, most of the CRN effectors related to the *Phytophthora*–citrus interaction remain without any functional characterization.

INTERACTIONS OF *CITRUS* AND PATHOGEN VECTORS

Phytophagous insects and mites play an important role in agriculture as pests or vectors of diverse pathogens. Similar to

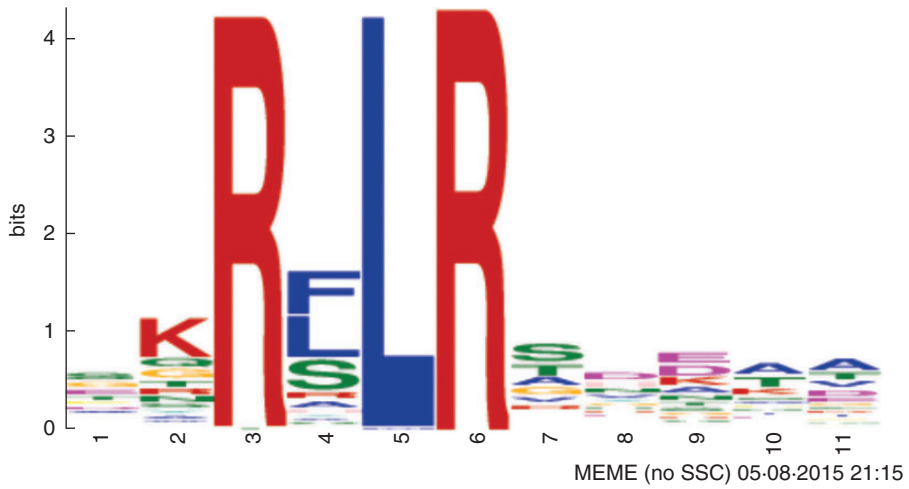

Fig. 9. Conserved RxRL domain in *P. parasitica* effectors.

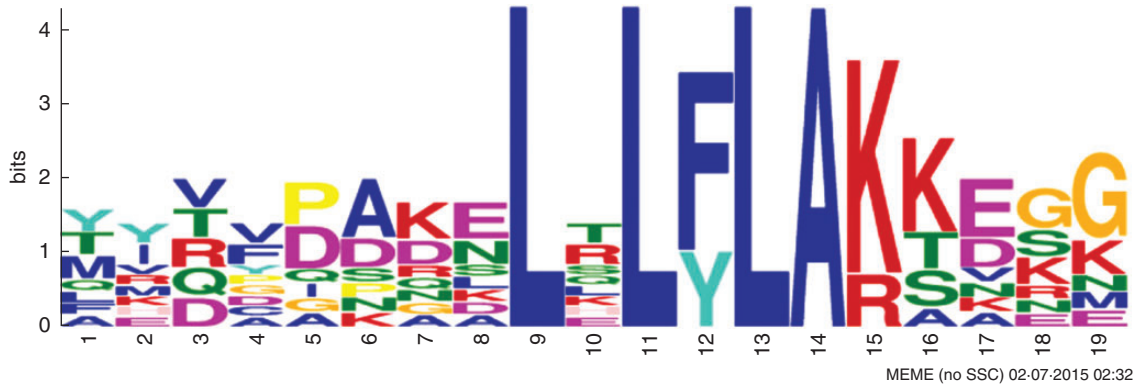

FIG. 10. Conserved LxLFLAK domain in *P. parasitica* CRN effectors.

many microbes, arthropods are also able to manipulate the physiological mechanisms of host plants (Stuart, 2015).

Plants recognize arthropod attacks by perception of HAMPs arising from wounds, contact, oral (regurgitate and saliva) and oviposition secretions. These then trigger signalling cascades similar to those characterized in a large range of plant–microbe interactions. The best known insect HAMPs are elicitors present in oral secretions, such as β-glucosidase, fatty acid-amino acid conjugates (FACs), glucose oxidase and inceptins (Sharma *et al*., 2014; Acevedo *et al*., 2015). These molecules promote activation of several plant defence responses, such as induction of plant calcium fluxes, activation of MAPK pathways, production of ROS, volatile emissions, and biosynthesis and signalling of plant defence hormones, such as jasmonic acid (JA), ethylene (ET) and salicylic acid (SA) (Wu and Baldwin, 2010).

During feeding, insects deliver their effectors through saliva, avoiding HAMP recognition. Insect saliva is composed of several important proteins, which facilitate entry and movement of the stylet of sap-sucking insects such as hydrolases, and perform detoxification of plant defence substances (superoxide dismutases, peroxidases and phosphatases) (Nicholson *et al*., 2012; Sharma *et al*., 2014). Calmodulin and/or other Ca^{2+} binding proteins are also present in insect saliva, preventing the occlusion of sieve-tubes caused by callose deposition or other innate plant responses (Will *et al*., 2007; Carolan *et al*., 2009; Hattori *et al*., 2012). Glucose oxidase (GOX), an effector present in the oral secretions of several insects, manipulates SA- and JA-related genes in different host plants (Zhu-Salzman *et al*., 2005; Hogenhout and Bos, 2011). Additionally, some effectors are able to induce chlorosis or repression of microbe elicitors (Bos *et al*., 2010; Elzinga and Jander, 2013), suppress plant protease inhibitors and other defence genes, or promote post-translational modifications (Acevedo *et al*., 2015), as well as repress wound-inducing responses (Consales *et al*., 2012).

Conversely, plant-associated microbes can manipulate plants and the physiology of their vectors to facilitate their transmission and dispersion (Felton and Tumlinson, 2008; Clark *et al*., 2010; Frago *et al*., 2012; Junker, 2014; Acevedo *et al*., 2015).

Since the transmission of many citrus diseases is facilitated by arthropods, here we provide some aspects of insect–microbe interactions for the major vectors associated with citrus crops.

Huanglongbing (HLB)

Diaphorina citri (Asian citrus psyllid, ACP) is the most important vector of HLB (Fig. 11), responsible for transmission of '*Ca*Las' bacteria across the Asian and American continents and transmission of '*Ca*Lam' in South America (Bové, 2014; Haapalainen, 2014).

Modifications of citrus volatile profiles caused by attack of this insect were reported (Hijaz *et al*., 2013), although until recently no candidate effectors were proposed for *D. citri*. However, in the genome of this insect there is a glucose oxidase-like gene and a putative secreted β-glucosidase without functional characterization, which are putative effectors of *D. citri*. This interference is based upon the characterization of these molecules as effectors in other insect species (Hogenhout and Bos, 2011; Consales *et al*., 2012).

Additionally, 86 miRNA sequences were found in the *D. citri* EST database. These sequences are phylogenetically related to 15 biotic stress-associated miRNA sequences of the Hessian fly (*Mayetiola destructor*), which are expressed in specific plant host genotypes. These observations led to the suggestion that these miRNAs may act on plant–insect interactions by regulating virulence factors (Khalfallah *et al*., 2015).

In contrast, '*Ca*Las' strongly manipulate insect physiology. The presence of '*Ca*Las' increased mortality of infected adult insects, delayed development of infected ACPs and increased fecundity (infected females laid more eggs than healthy females) (Pelz-Stelinski and Killiny, 2016). '*Ca*Las'-infected ACP insects have decreased expression of detoxification genes such cytochrome P_{450}, esterases and glutathione transferases (Tiwari *et al*., 2011*b*). Repression of these genes promotes loss of fitness and increases susceptibility to insecticides (Tiwari *et al*., 2011*a*, *b*, *c*). '*Ca*Las' infection also interferes with the metabolism and immune system of *D. citri*, manipulating free amino acid availability, iron transport and cytoskeleton networks, in addition to suppressing 90 % of the immune genes at immature stages (Fisher *et al*., 2014; Vyas *et al*., 2015). Down-regulation of insect immune genes may be associated with expression of an as yet uncharacterized gene '*Ca*Las', containing an imelysin-like domain. In other bacterial species, homologues of this gene act to suppress the immune systems of insects (Yan *et al*., 2013). In addition, '*Ca*Las' induces modification of plant volatiles making them more attractive to *D. citri*

Fig. 11. (A) Nymphs and (B) adults of *Diaphorina* citri, the vector of *Candidatus* Liberibacter spp., the causal agent of HLB.

Fig. 12. *Macugonalia leucomelas*, one of the sharpshooter vectors of CVC disease.

and allowing bacterial dispersal (Mann *et al*., 2012; Hijaz *et al*., 2013).

Citrus variegated chlorosis

Xylem sap-feeding insects belonging to the families Cicadellidae and Cercopidae (Fig. 12) are the major vectors of the bacteria *X. fastidiosa* (Almeida *et al*., 2013). Until recently, no putative effector molecules were described for these insects during interactions with *X. fastidiosa*. However, some genes were characterized as being important for this interaction.

Gene expression analysis revealed that *Homalodisca coagulata* promotes lower activation of stress-related genes than mechanical wounds in citrus plants, suggesting the presence of

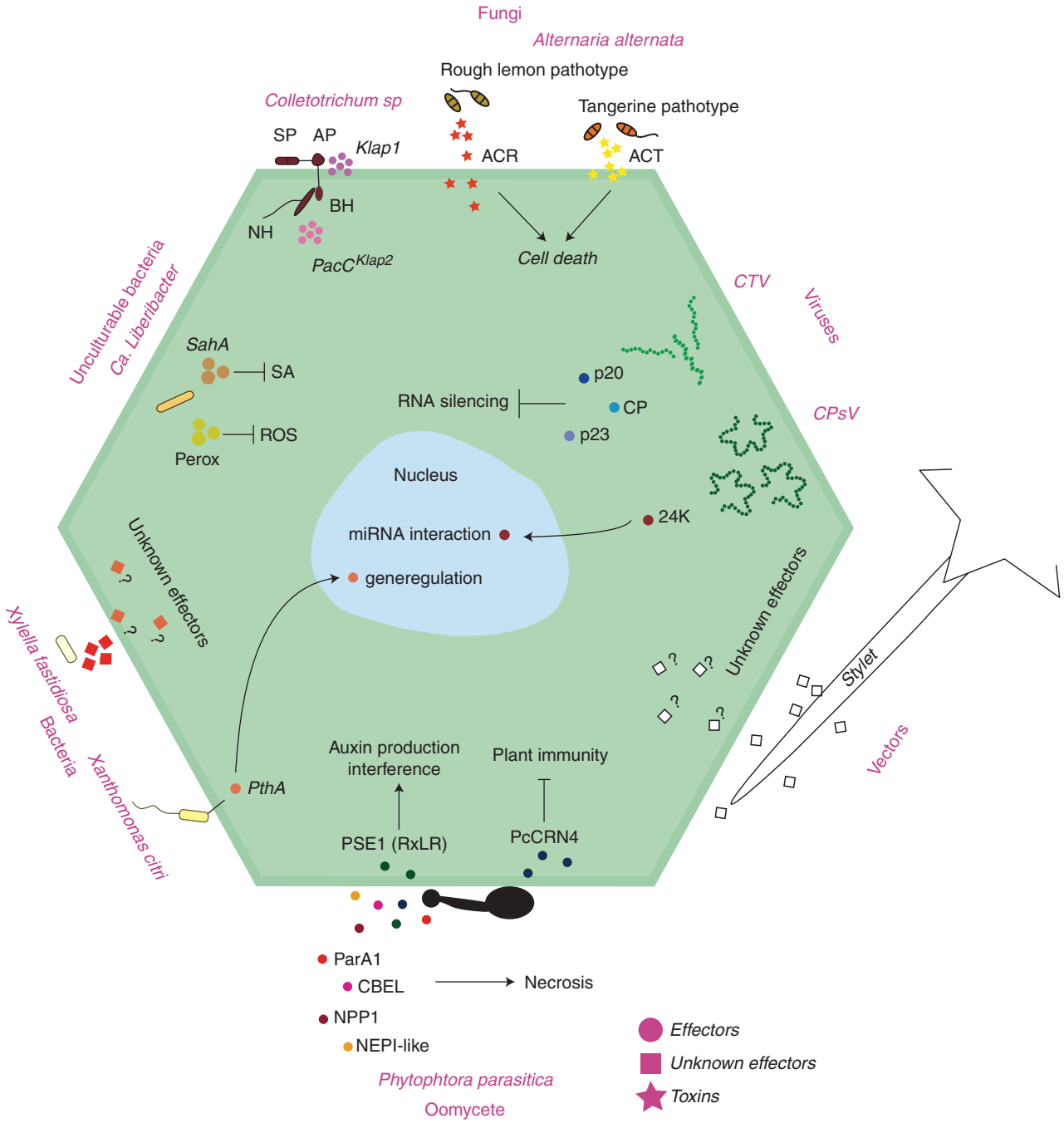

FIG. 13. Schematic representation of a citrus cell and its general molecular interactions with the main fungi, viruses, vectors, oomycetes, bacteria and unculturable bacteria. **Fungi:** The infection process of *C. acutatum* requires proteins *Klap 1* for appressorium development and infection on leaves of key lime. Proteins *PacC*^{KLAP2} are required for pH adjustment in the transition from biotrophic hyphae to necrotrophic. Spore (SP), appressorium (AP), biotrophic hyphae (BH), necrotrophy hyphae (NH). The conidia produced by both pathotypes of *A. alternata* germinate quickly and begin to produce toxins. The putative target site of ACT-toxins in tangerine is the plasma membrane and of ACR-toxin is the mitochondrion, both leading to cell death. **Virus:** *Citrus tristeza virus* (CTV) deploys three effectors – p20, p23 and coat protein (CP) – with activities of viral suppressors of RNA silencing (VSR) to overcome host resistance. *Citrus psorosis virus* (CPsV) 24K protein physically interacts with pre-miRNA in the nucleus and induces the expression of genes involved in disease symptom development, suggesting a putative VSR function. **Vectors:** During feeding, vectors deliver putative effectors through saliva in stylets into the plant intercellular space which can modulate host plant recognition, volatile emission and defence. **Oomycetes:** Oomycete pathogens of citrus can secrete two types of proteins: apoplastic and cytoplasmic effectors. The apoplastic effectors, such as NEP-like, ParA1, NPP1 and CBEL, are frequently related to plant responses and necrosis elicitation. The cytoplasmic effectors, such as PSE1 (RxLR effector) and PcCRN4 (Crinkler effector), may interfere with the physiology of plants (auxin production) or suppress plant immunity, respectively. **Bacteria:** *X. citri* is able to inject into the plant host cell effectors such as PthA, which heads towards the cell nucleus where it controls gene regulation. *Xylella fastidiosa* may have effector molecules that are secreted into the plant, but none have been characterized to date. **Unculturable bacteria:** '*Candidatus* Liberibacter asiaticus' might quench H_2O_2 accumulation and signalling events by secretion of peroxidase enzyme during early stages of infection. A functional salicylate hydroxylase (SahA) predicted in the '*Ca*Las' genome converts salicylic acid (SA) into catechol and might suppress SA-mediated defence.

an insect elicitor which modulates plant response (Mozoruk *et al*., 2006).

Salivary β-glucosidase of *Homalodisca vitripennis* is important for transmission of *X. fastidiosa*, digesting bacterial biofilm in the Pierce's disease pathosystem (Backus *et al*., 2012, 2015). The action of this enzyme may also be important for CVC disease, since biofilm formation is critical for establishment and transmission of *X. fastidiosa* through its host.

Many studies of Pierce's disease of grapevines have demonstrated that initial adhesion and retention of *X. fastidiosa* of its insect vector are related to fimbrial and afimbrial adhesins (such *Hxf*, *XadA*, and *fimA* genes), as well as LPS (Killiny and Almeida, 2009, 2014; Killiny *et al*., 2012; Orlovskis *et al*., 2015; Rapicavoli *et al*., 2015). This colonization is dependent on a quorum-sensing mechanism composed of components of the *rfp* cluster (regulation of pathogenicity factors), which senses a diffusible signalling factor (DFS) and alters the expression of many genes involved with attachment and biofilm formation, including *hxfA*, *hxfB* and *fimA* (Baccari *et al*., 2014; Ryan *et al*., 2015).

The extracellular polysaccharides (EPS) and outer membrane vesicles of *X. fastidiosa* also play a role in retention and transmission of this pathogen by insect vectors (Killiny *et al*., 2013; Ionescu *et al*., 2014).

Citrus tristeza disease

Citrus tristeza disease is vectored by the brown citrus aphid (*Aphis citricidus*). The majority of insect effectors characterized so far belong to aphid family members, but only two of these molecules were identified from *A. citricidus*. The first is a protein, C002, which has no functional characterization, but plays an important role in the feeding behaviour of pea aphid (*Acyrthosiphon pisum*), reducing the time of contact of this insect with plant sieve elements when this gene is suppressed (Mutti *et al*., 2008). Expression of C002 homologues of *Myzus persicae* in *N. benthamiana* enhances aphid fecundity (Bos *et al*., 2010). These data support the hypothesis that *A. citricidus* C002 may be important for the interaction of this aphid with its plant host. The second *A. citricidus* putative effector is a cysteine-rich protein that was first identified in *A. pisum* and is present across many aphid species (Guo *et al*., 2014). This protein is highly expressed in salivary glands and in the first instar of *A. pisum*; however, knockdown of this gene does not interfere with survival or feeding (Guo *et al*., 2014).

Other important arthropods

Mites, along with insects, are the most important agricultural pests in the arthropod group. In citrus crops, the mite *Brevipalpus yothersi* is notable for being a vector of citrus leprosis virus (CiLV) but no effector molecules have yet been characterized. Evidence of plant volatile and defence manipulation by mites has been reported in the literature (Albarouki and Deising, 2013; Zhurov *et al*., 2014; Alba *et al*., 2015; Martel *et al*., 2015; Godinho *et al*., 2016). In a recent study, three salivary effectors were identified for two mite species: *Tetranychus urticae* and *Tetranychus evansi* (Villarroel *et al*., 2016). These proteins were described acting on modulation of JA and SA responses and increasing mite performance.

OVERVIEW, CONCLUSIONS AND PERSPECTIVES

As perennial woody plants, citrus are in constant interaction with various abiotic and biotic factors, including viroids, viruses, mollicutes, bacteria, oomycetes and fungi. Many of the pathosystems are highly complex and require a deeper understanding to enable development of suitable disease control strategies.

The present review summarizes important information available regarding PAMPs, PRRs, effectors and R-genes associated with the main interactions of citrus species and their pathogens and insect vectors (Fig. 13). Whilst gaps still remain, available information is potentially useful in the development of disease-resistant plants through both conventional breeding programmes and biotechnology-based approaches, which include the development of transgenic or cisgenic citrus, genome editing and host induced gene silencing (HIGS). We have not included in this review those citrus pathogens that still lack robust information regarding molecular interaction with their hosts; however such information will probably be available in the near future.

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