Molecular Basis of *Citrus sunki* Susceptibility and *Poncirus trifoliata* Resistance Upon *Phytophthora parasitica* Attack

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Coevolution has shaped the molecular basis of an extensive number of defense mechanisms in plant-pathogen interactions. *Phytophthora parasitica*, a hemibiotrophic oomycete pathogen and the causal agent of citrus root rot and gummosis, interacts differently with *Citrus sunki* and *Poncirus trifoliata*, two commonly favored citrus rootstocks that are recognized as susceptible and resistant, respectively, to *P. parasitica*. The molecular core of these interactions remains elusive. Here, we provide evidence on the defense strategies employed by both susceptible and resistant citrus rootstocks, in parallel with *P. parasitica* deployment of effectors. Time course expression analysis (quantitative real-time polymerase chain reaction) of several defense-related genes were evaluated during i) plant disease development, ii) necrosis, and iii) pathogen effector gene expression. In *C. sunki*, *P. parasitica* deploys effectors, including elicitors, NPP1 (necrosis-inducing *Phytophthora* protein 1), CBEL (cellulose-binding elicitor and lectin activity), RxLR, and CRN (crinkler), and, consequently, this susceptible plant activates its main defense signaling pathways that result in the hypersensitive response and necrosis. Despite the strong plant-defense response, it fails to withstand *P. parasitica* invasion, confirming its hemibiotrophic lifestyle. In *Poncirus trifoliata*, the effectors were strongly expressed, nevertheless failing to induce any immunity manipulation and disease development, suggesting a nonhost resistance type, in which the plant relies on preformed biochemical and anatomical barriers.

Citrus fruit and juice are consumed worldwide and are produced in more than 140 countries, with China (24.4%), Brazil (15.64%), and the United States (7.75%) representing the main

producers (FAO 2015). Over the years, however, the citrus industry has been suffering the impact of many biotic and abiotic stress conditions. Several diseases caused by a diverse range of viruses, bacteria, fungi, oomycetes, and nematodes have led to the annual loss of millions of dollars, which globally compromises the economy and food security (Dalio et al. 2017). In addition to the use of chemical defensives and fertilizers, a frequent management strategy in citrus against different stresses is the use of suitable rootstocks, such as ‘Sunki’ mandarin (*Citrus sunki* Hort. Ex Tan.) and trifoliolate orange (*Poncirus trifoliata* (L.) Raf. cv. Rubidoux). *C. sunki* rootstocks have demonstrated tolerance to drought, salinity, citrus tristeza disease, citrus blight, and citrus sudden death, whereas *Poncirus trifoliata* has been tolerant to low temperatures, nematodes, *Phytophthora* spp., as well as citrus tristeza disease and citrus sudden death (Boava et al. 2011). Accordingly, both species have been extensively used in genetic breeding programs for the production and selection of hybrids, known as citrandarins, which possess the desired features of the parental cultivar (Siviero et al. 2006).

Microbes maintain a long-standing interaction with plants, which may be either a parasitic or mutualistic relationship (Cuming 2009). During evolution, the molecular basis of plant-microbe interactions has constantly changed and has molded the mechanisms of plant defense (Cuming 2009; Gilbert and Parker 2016). These dynamic changes include both immediate and long-term responses, such as transcriptional gene reprogramming, production of metabolites, and genome stability and epigenetic alterations (Boyko and Kovalchuk 2011). At the front line, plant defenses rely on the structural and chemical barriers that are constitutively produced to prevent pathogen entrance (Malinovsky et al. 2014). At the second line, plants set a complex network of signaling pathways that encompass microbe perception and subsequent defense activation (Zhang et al. 2013).

A coevolutionary model of plant-pathogen interaction has been established that depicts different layers of plant immune response and pathogen attack (Jones and Dangl 2006). In the first layer, plants perceive conserved pathogen-associated molecular patterns (PAMPs) by their pattern recognition receptors (PRRs) and induce PAMP-triggered immunity (PTI). Generally, PTI results in an influx of Ca^{2+} into the plant cytosol (known as a Ca^{2+} burst), production of extracellular reactive oxygen species (ROS), protein phosphorylation, protein kinases activation, and modulation of defense-related phytohormones, such as salicylic acid (SA), jasmonate (JA), and ethylene (ET) (Bigear et al. 2015). Successful pathogens are able to overcome PTI by secreting

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effector molecules into one or both the apoplast and cytoplasm of the host plant cells (Cui et al. 2015). The effector molecules modulate the plant physiology and innate immunity, which leads to a state known as effector-triggered susceptibility (ETS) (Kazan and Lyons 2014; Lo Presti et al. 2015). Therefore, pathogens and effectors play an important role as selective agents in host plant populations (Chisholm et al. 2006; Karasov et al. 2014).

Plants with resistance (R) genes, whose products recognize the pathogen effectors, are able to acquire an immunity condition named effector-triggered immunity (ETI) (Thomma et al. 2011). During ETI, plant defenses are prolonged in comparison with the responses triggered by PTI, being generally characterized by plant cell death surrounding the infection site, known as the hypersensitive response (HR), and induction of systemic acquired resistance (SAR) in host plants (Kushalappa et al. 2016). These pathogen effectors are fast-evolving molecules, which results in derivative forms of an 'ancient' effector; consequently, recently evolved effectors may either gain new molecular activities, no longer be impaired, or both by a host R protein, presented in a previous resistance plant (Marín et al. 2013). This evolutionary event generates another layer of ETS that continuously evolves in a dynamic way (Cui et al. 2015).

During pathogenic infection, distinct phytohormones can be induced and act as signal molecules to trigger the production of antimicrobial compounds (Verma et al. 2016) in an intricate, orchestrated, and interactive network (Verhage et al. 2010). Typically, plants produce SA, JA, and ET as major defense signals upon pathogen attack (Robert-Seilanianz et al. 2011); however, plants also deploy other mechanisms to counteract pathogen attack, and such strategies include the production of a range of phenolic compounds, defensins, protease inhibitors, hydrolytic enzymes, ROS, and phytoalexins (Ahuja et al. 2012; Hara-Nishimura and Hatsugai 2011; Kim et al. 2009; Lacerda et al. 2014; Tripathy and Oelmüller 2012).

Several *Phytophthora* species threaten citrus production worldwide, and the most important species are *P. citricola* Sawada, *P. citrophthora* (R. E. Sm. & E. H. Sm.) Leonian, *P. drechsleri* Tucker, *P. palmivora* (E. J. Butler) E. J. Butler, and *P. parasitica* Dastur (= *P. nicotianae*). Due to its vast distribution and pathogenicity, *P. parasitica* is considered the main citrus oomycete pathogen, causing citrus gummosis and citrus root rot diseases (Panabieres et al. 2016). *P. parasitica* is an aggressive soil-borne pathogen with a fast growth rate and hemibiotrophic lifestyle and rapidly colonizes roots and stems of susceptible citrus species.

Among citrus, *C. sunki* is a highly susceptible rootstock, whereas *Poncirus trifoliata* is considered resistant or tolerant to *P. parasitica* (Boava et al. 2011); however, the physiological and molecular mechanisms behind citrus susceptibility or tolerance are still poorly characterized. Cytological studies have showed extensive pathogen colonization in roots and leaves of susceptible *Arabidopsis thaliana*, with formation of abundant haustoria-like structures (Attard et al. 2014; Wang et al. 2011). *A. thaliana* mutant lines with compromised SA, JA, and ET signaling pathways have shown increased susceptibility to *P. parasitica*, demonstrating the relevance of these phytohormones to plant defense and suggesting that components of phytohormone signaling pathways may be potential targets of pathogenic effectors. Accordingly, Evangelisti et al. (2013) reported that the *P. parasitica* RxLR penetrating specific effector 1 (PSE1) targets auxin-related signals to impair *A. thaliana* physiology, thus enabling infection. Attard et al. (2014) found that RxLR effectors are differentially expressed during the necrotrophic stage of infection in *A. thaliana*.

To date, several apoplastic effectors have already been reported to modulate citrus physiology (Khatib et al. 2004; Obwald et al. 2014), including the elicitor ParA1 (Kamoun et al. 1993), the

necrosis and ethylene-inducing peptide (NEP1-like protein), the necrosis-inducing *Phytophthora* protein 1 (NPP1) (Fellbrich et al. 2002), and the cellulose-binding elicitor and lectin activity (CBEL) effectors. However, the expression dynamics of cytoplasmic effectors such as RxLR and crinklers (CRN) are still to be determined during citrus-*P. parasitica* interaction.

Here, we aimed to address the following scientific questions. i) Does the pathogen colonize both *C. sunki* and *Poncirus trifoliata* tissues? ii) Do both citrus species recognize the pathogen attack and activate their defense systems? iii) If so, which genes and signaling pathways are activated by each species? iv) Does *P. parasitica* deploy different sets of effectors depending on the host? Hence, understanding the molecular interactions and finding the key components that mediate *Poncirus trifoliata* tolerance and *C. sunki* susceptibility may contribute to the development of alternative strategies to control *P. parasitica*-inducing disease, such as targeted genetic breeding, host-induced gene silencing, and cisgenic plants.

RESULTS

C. sunki and *Poncirus trifoliata* exhibit contrasting responses upon *P. parasitica* infection.

Seedlings of *C. sunki* and *Poncirus trifoliata* were inoculated with *P. parasitica* zoospores and disease progression was examined. *C. sunki* plants developed severe symptoms of leaf wilt and root rot at 96 h postinoculation (hpi); in contrast, no symptoms were observed in *Poncirus trifoliata* seedlings (Fig. 1A to C). To confirm that infection was effective in both species despite the lack of symptoms in *Poncirus trifoliata*, we were able to re-isolate *P. parasitica* from *C. sunki* and *Poncirus trifoliata* roots (Fig. 1D and E), indicating the presence of either viable reproductive structures, hyphae, or both on root tissues.

Indeed, *P. parasitica* mycelia were visualized in the root cortex and sporangia were observed, as well, in roots of *C. sunki* (Fig. 2). These observations were consistent with the high levels of *P. parasitica* DNA present in *C. sunki*-inoculated roots (Fig. 3B). *P. parasitica* growth in the root tissues of *C. sunki* was confirmed by linear regression statistical analysis (Fig. 3C). On the other hand, none of *P. parasitica* structures were observed in *Poncirus trifoliata* seedlings under light microscopy (Fig. 2B, D, and F), although *P. parasitica* genomic DNA still could be retrieved from infected roots but decreased over time (Fig. 3C). No hyphae, mycelium, or *P. parasitica* DNA were found in control plants (Figs. 2 and 3).

This opposite growth behavior of *P. parasitica* in each citrus species is thoroughly represented in Figure 3, illustrating the amount of *P. parasitica* genomic DNA measured in both species, showing an increase in *C. sunki* and a decrease in *Poncirus trifoliata* roots over the time-course experiment. These findings assure the assumption of *C. sunki* susceptibility and *Poncirus trifoliata* resistance to *P. parasitica* infection.

SA-, JA-, and ET-mediated defenses do not contribute to citrus resistance against *P. parasitica*.

To gain insights into the defense mechanisms of susceptible (*C. sunki*) and resistant (*Poncirus trifoliata*) citrus plants upon *P. parasitica* infection, we evaluated the expression of genes related to the major plant defense pathways, i.e., SA, JA, and ET, in addition to genes related to basal immunity and other physiological processes.

Quantitative real-time polymerase chain reaction (RT-qPCR) analyses were carried out in *P. parasitica*-infected roots sampled at 3, 6, 12, 24, 48, and 96 hpi (Figs. 4, 5, and 6; Supplementary Figs. S1 through S7). *P. parasitica* infection induced transcriptional reprogramming of the major defense pathways in *C. sunki* (Figs. 4 and 6). Most of the genes exhibited slight

increases at 3 and 6 hpi, returning to basal expression level at 12 hpi, then a peak of upregulation at 24 hpi, followed with a slight upregulation 48 hpi onwards. The majority of SA-related genes, which were herein evaluated, showed induction during all sampled timepoints, with the greatest changes observed for *Heat Shock Protein90 (HSP90)* and *REQUIRED FOR MLA12 RESISTANCE 1 (RAR1)* genes. Similar patterns were detected for genes that participate in JA and ET signaling pathways (Fig. 6) and for *R* genes and genes related to oxidative burst and HR, which were all also up-regulated. Despite the differential expression of plant immunity-related genes, *Aquaporin1* gene expression (strongly associated with physiology of healthy plants) did not alter at early timepoints and only showed slight downregulation at 96 hpi, which indicates that the plant immune-gene expression could be independent of the normal physiologic gene expression. Likewise, we tested five candidate housekeeping genes for both citrus species during infection, and these all received high scores, on NormFinder and geNorm software programs (Máximo et al. 2017), to also indicate a detached expression pattern of defense-related genes and normal physiologic genes. Intriguingly, *Poncirus trifoliata* exhibited constitutive levels for most of the defense-related genes that were evaluated (Fig. 5), suggesting that either this citrus species does not recognize this pathogenic organism or this plant defense resistance does not involve activation of those immunity-related genes.

Generally, plants face a trade-off between growth and defense. After pathogen recognition, plants close stomata, which decreases water uptake and photosynthesis, reallocating most of the energy supply to fight the pathogenic attack (Gudesblat et al. 2009). Here, the water uptake rates decrease in a linear pattern for both citrus species, but in *C. sunki*-infected plants, the water uptake rate is 0.91% higher than in *C. sunki* control plants (*t* test *P* value, 0.0049). In *Poncirus trifoliata*, an inverse behavior was observed, with infected plants showing statistically significant difference of −0.80% compared with non-inoculated plants (*t* test *P* value, 0.0467379). *Poncirus trifoliata* actively decreased its water consumption from 24 hpi onwards (Fig. 7), indicating that, in spite of the lack of defense-related gene activation, *Poncirus trifoliata* was able to recognize *P. parasitica* attack and adjusted its own physiology. On the other

hand, *C. sunki* increased water uptake at 24 hpi and maintained its consumption above control plants. This result can be explained either by decreased hydric potential in *C. sunki* roots, due to the release of more solutes to the tissues after pathogen infection and tissue necrosis, or by the action of pathogen effectors that manipulate plant hormone signaling pathways (Evangelisti et al. 2013).

***C. sunki* produces ROS for defense against *P. parasitica*.**

Several genes related to oxidative burst and HR were up-regulated in *C. sunki*-infected plants. To check if this increase would, indeed, result in release of ROS and, consequently, oxidative burst, we performed diaminobenzidine tetrahydrochloride (DAB) staining in root samples, infected or not, of both plant species. DAB is oxidized by hydrogen peroxide and generates a dark brown precipitate—the browner the stains in the tissues, the more ROS were released. Thus, *C. sunki*-infected roots appeared darker than *Poncirus trifoliata*-infected roots, as did both infected tissues compared with noninfected control plants (Fig. 8).

The DAB assay confirmed our hypothesis that a strong increase of defense-related genes would result in oxidative burst and, ultimately, necrosis (Fig. 1B). This robust plant defense response would be an effective strategy against biotrophic pathogens; however, it was not sufficient to prevent *P. parasitica* growth and colonization (Fig. 3), most likely because *P. parasitica*, as a hemibiotrophic pathogen, is well-adapted to both “trophic” conditions.

Putative *P. parasitica* effector genes are expressed during interaction with *C. sunki* and *Poncirus trifoliata*.

It could also be speculated that the root microenvironment, including root exudates and cell-wall fragments, could interfere with effector expression of *P. parasitica*, resulting in differences in susceptibility and resistance found for the plants. We tested the hypothesis that *P. parasitica* interacts with *Poncirus trifoliata* by secreting few effectors in a mild intensity, while *C. sunki* would be overwhelmed with higher levels of the pathogen effectors. To shed light on this issue, we evaluated the expression of several *P. parasitica* candidate effectors, either

Fig. 1. *Citrus sunki* and *Poncirus trifoliata* plants, infected with *Phytophthora parasitica* and control noninfected, sampled at 96 h postinoculation. **A**, Wilt leaves in infected *C. sunki*. **B** and **C**, Roots of infected *C. sunki* and *Poncirus trifoliata*. Arrows and circles indicate mycelia and root rot, respectively. **D** and **E**, Plates containing selective medium to reisolate *P. parasitica* from *C. sunki* and *Poncirus trifoliata*.

apoplastic or cytoplasmic, during interaction with both citrus species.

Most candidate effector genes were up-regulated in both plants, which rejects our initial hypothesis (Figs. 9 and 10); however, their

expression pattern was distinct between samples from each of the infected plants, as we expected. In *C. sunki*, some gene expressions were decreased, e.g., *PpCRN4*, which was down-regulated at all timepoints, and *PpCRN8*, which had decreased expression at 6

Fig. 2. A to F, *Phytophthora parasitica*-infected plants sampled at 96 h postinoculation and G to H, noninoculated control plants. E, Magnified view of infected *C. sunki*, with arrows indicating *P. parasitica* hypha. F, Magnified view of infected *Poncirus trifoliata* without apparent oomycete growth. Longitudinal sections of *Citrus sunki* root tips are shown in A, C, E, and G and of *Poncirus trifoliata* in B, D, F, and H. Samples were stained with toluidine blue and were observed under light microscope.

Fig. 3. A, Color scale of genomic DNA content. **B**, *Phytophthora parasitica* genomic DNA content in *Citrus sunki* and *Poncirus trifoliata* roots. **C**, Linear regression of the logarithm base 2 of the amount of *P. parasitica* genomic DNA (in micrograms per gram of fresh weight [FW]) in *C. sunki* and *Poncirus trifoliata* roots sampled at 3, 6, 12, 24, 48, and 96 hpi. Notice the increasing growth rate in *C. sunki* plants and a steadily decreasing rate in *Poncirus trifoliata*. Both relationships are highly statistically significant according to *P* values.

Fig. 4. Hierarchical clustering of gene expression in *Phytophthora parasitica*-infected *Citrus sunki* plants. The heat map illustrates the fold changes in gene expression ($\log_2$ scale) in samples collected at 0, 3, 6, 12, 48, and 96 h postinoculation (hpi). Different shades represent induced or repressed gene expression. Biological and technical triplicates were used in the analysis. Gene acronyms are listed in the column at the right.

and 24 hpi (Fig. 9). Apart from *PpCRN4*, *PpCRN8*, and *PpELI3*, the other candidate effector genes were highly up-regulated at the later timepoints, when the infection was already established. Differently, in *Poncirus trifoliata*, the vast majority of *P. parasitica* effector genes, with the exception of *PpELI3*, were substantially induced at most of the timepoints, suggesting that *P. parasitica* actively secreted these effectors early in the infection, either as an attempt to break *Poncirus trifoliata* defense barriers or to manipulate the plant immune system (Fig. 10).

DISCUSSION

Deciphering the molecular mechanisms of pathogen effector deployment to favor infection in parallel with plant counter-defense strategies to avoid disease is a major focus of the plant-microbe interaction research community. Frequently, plants can rapidly activate their defense system upon recognition of PAMPs or effectors and overcome disease development; however, this is not a rule. In the present study, we analyzed two citrus species with contrasting responses to *P. parasitica* infection (*C. sunki*, highly susceptible, and *Poncirus trifoliata*, resistant) and how their physiology is modulated under the influence of pathogen invasion and effector induction. Surprisingly, we found that the susceptible plant (*C. sunki*) rapidly recognizes *P. parasitica* invasion and strongly activates several genes related to SA, JA, and ET defense signaling pathways. Although robust, this defense boost was not sufficient to cope with *P. parasitica* invasion and prevent development of disease symptoms. On the other hand, *Poncirus trifoliata* did not show activation of any defense signaling pathway, although this citrus plant remains resistant to *P. parasitica* colonization, without development of disease symptoms.

A repertoire of genes related to defense response was therefore evaluated in susceptible and resistant citrus plants upon *P. parasitica* infection. Overall, most of the genes were induced

in *C. sunki*, whereas only a few genes were differentially expressed in *Poncirus trifoliata*. We noticed that *C. sunki* responds with two tiers of defense against *P. parasitica*. First, a slight induction of defense genes early, at 3 hpi, is observed, followed by a basal gene expression at one or both 6 and 12 hpi, and second, a strong upregulation of these genes later, at 24 hpi. We have speculated that the first tier of defense might be mainly induced by general PAMPs, just after initial contact between plant and pathogen. Generally, a large number of defense-related genes are up-regulated following the perception of PAMPs by the PRRs present in the plant cell membranes (Li et al. 2016). Early-induced genes include those mainly related to the SA signaling pathway and defense responses (Lewis et al. 2015). This is consistent with the gene expression pattern observed in Figures 4 and 5.

Most of the evaluated genes related to the SA signaling pathway were up-regulated upon *P. parasitica* infection, except the *RAR1* gene, which was negatively regulated (Fig. 6). *RAR1* is a component required for induction of the coiled coil–nucleotide binding site (NBS) type resistance genes and further signaling responses (Song et al. 2013). The knockout *rar1* mutants are associated with limited ROS-induced programmed cell death (PCD) and with compromised basal resistance against fungi and bacteria pathogens. In this case, suppression of *RAR1* might contribute to diminished ROS production and its effect on the pathogen colonization (Kaurilind et al. 2015). Furthermore, *rar1* mutants exhibit lower accumulation of immune receptors than wild-type plants (Bieri et al. 2004). *HSP90*, otherwise, was the most up-regulated gene in our analyses; *HSP90* encodes a highly conserved and widely distributed chaperone, which, among other activities, mediates a signal transduction pathway that is linked to disease in plants (Xu et al. 2012). The complex formed by the interaction among HSP90, RAR1, and SUPPRESSOR OF THE G2 ALLELE OF SKP1 (SGT1) modulates the activation and stability of R proteins and contributes to the recognition of

Fig. 5. Hierarchical clustering of gene expression in *Phytophthora parasitica*-infected *Poncirus trifoliata* plants. The heat map illustrates the fold changes in gene expression ($\log_2$ scale) in samples collected at 0, 3, 6, 12, 48, and 96 h postinoculation (hpi). Different shades represent induced or repressed gene expression. Biological and technical triplicates were used in the analysis. Gene acronyms are listed in the column at the right.

Fig. 6. Gene regulatory network of defense-related genes. **A**, *Citrus sunki* and **B**, *Poncirus trifoliata* plants upon *Phytophthora parasitica* infection. Genes were grouped into salicylic acid (SA), jasmonate, and ethylene pathways and oxidative burst-, defense-, and physiology-related genes. Different shades represent induced or repressed gene expression. Notice upregulation for most of the genes in *C. sunki*, in particular, SA-related genes, and a fair constitutive gene expression in *Poncirus trifoliata*.

Fig. 7. Linear regression showing water uptake rate in *Phytophthora parasitica*-infected *Citrus sunki* and *Poncirus trifoliata* sampled at 24, 48, 72, and 96 h postinoculation. **A**, Water uptake is higher in infected *C. sunki* compared with noninfected control plants, showing a statistically significant average difference of 0.91% (adjusted *P* value, 0.0049). **B**, *Poncirus trifoliata* has an inverse behavior presenting a statistically significant average difference of -0.80% (adjusted *P* value, 0.0467379). In both plants, the water uptake decreases in a linear pattern over time.

pathogen effectors (Ito et al. 2015). On the other upstream branch of the SA pathway, *ENHANCED DISEASE SUSCEPTIBILITY 1* (*EDS1*) and *PHYTOALEXIN DEFICIENT 4* (*PAD4*) exhibited a steady-state expression over the course of the infection. These

genes function along with *SENESCENCE-ASSOCIATED GENE 101* (*SAG101*) as a complex that is required for regulating the expression of toll interleukin 1 receptor–NBS type resistance genes (Wagner et al. 2013).

Fig. 8. A, Hydrogen peroxide (H_2O_2) detection by diaminobenzidine tetrahydrochloride staining in *Citrus sunki* and *Poncirus trifoliata* roots infected with *Phytophthora parasitica* and sampled at 96 h postinoculation. B and C, Magnified view of root tips of B, a control sample, C, *P. parasitica*–inoculated *C. sunki*, D, a control sample, and E, *Poncirus trifoliata*–inoculated. Sc = *C. sunki* control, Si = *C. sunki*–inoculated, Tc = *Poncirus trifoliata* control, and Ti = *Poncirus trifoliata*–inoculated.

Fig. 9. Hierarchical clustering of gene expression of *Phytophthora parasitica* effectors during interaction with *Citrus sunki* at 0, 3, 6, 12, 24, 48, and 96 h postinoculation (hpi). The heat map illustrates fold change in expression ($\log_2$ scale). Different shades represent induced or repressed gene expression. Apoplastic effectors (PpNPP1.1, PpNPP1.3, PpNPP1.4, PpELI2, PpELI3, PpCBEL1, PpCBEL2, PpCBEL3, PpCBEL4) and cytoplasmic effectors (PpRxLR1, PpRxLR17, PpCRN4, PpCRN8) were evaluated.

Genes related to SA biosynthesis and metabolism, such as *ISOCHORISMATE SYNTHASE 1 (ICS1)*, *SAR DEFICIENT 1 (SARD1)*, *BENZOIC ACID/SALICYLIC ACID CARBOXYL METHYLTRANSFERASE 1 (BSMT1)*, and *SA CARBOXYL METHYLTRANSFERASE (SABATH)*, had a similar gene expression profile, with a slight upregulation at 3 and 24 hpi (Fig. 6). Among them, *BSMT1* and *SABATH* were the most-induced genes, and both encode for methyltransferase proteins with activity over SA (Hippauf et al. 2010). The derivative methyl salicylate, produced by methyltransferase activity, can be transported to distal tissues through the phloem and it activates a systemic defense response, after being converted into SA by enzymes, such as *SABATH*, and triggering downstream SA signaling (Fu and Dong 2013). Therefore, a defense response associated to SAR might be active in *C. sunki* upon *P. parasitica* infection.

Altogether, the defense-related genes that act downstream in the SA signaling pathway, which includes transcription factors, pathogenesis-related proteins, and NBS genes, showed as up-regulated during infection (Fig. 6). This indicates that *P. parasitica* induces general defense mechanisms mediated by SA; however, this phytohormone signaling pathway is not sufficient to promote *C. sunki* resistance.

Furthermore, we also evaluated key genes related to JA and ET signaling pathways, as these phytohormones, in conjunction with SA, act in a crosstalk network that regulates plant defenses. A similar expression profile of JA- and SA-related genes was observed. Additionally, a consistent regulation of *CORONATINE INSENSITIVE 1 (COI1)* expression, which negatively regulates *JASMONATE-ZIM-DOMAIN PROTEIN 2 (JAZ2)* and consequently induces *MYC2* expression (Turner et al. 2002), was here observed. *MYC2* modulates a range of JA-dependent functions, including the regulation of defense mechanisms against pathogens and herbivores (Dombrecht et al. 2007). For ET-related gene expression, we detected an upregulation of most genes that are upstream in this signaling pathway. However, the gene *ETHYLENE-INSENSITIVE3-LIKE 1 (EIL1)*, which encodes a transcription factor, was repressed during infection. *EIL1* coordinates several ET responses, including plant development and defense responses (An et al. 2010).

We also evaluated genes that are associated with general defense responses, for instance, *Callose Synthase*, *GAMMA VACUOLAR PROCESSING ENZYME (GVPE)*, *LESION SIMULATING DISEASE 1 (LSD1)*. All these genes were induced, following the same profile of two waves of upregulation. *GPVE* is a vacuolar processing enzyme that is up-regulated under stress conditions and is associated to cell death (Qiang et al. 2012). *LSD1* regulates cell death by interacting with catalases that control the level of ROS into cells (Coll et al. 2011). These findings confirm that *C. sunki*

plants activate general defense mechanisms upon infection; however, these mechanisms do not contribute to plant resistance.

In contrast, *Poncirus trifoliata* defense-gene transcripts were weakly detected upon *P. parasitica* infection. We have speculated that the pathogen may not be adapted to surpass preformed physical and biochemical barriers and, thus, may be unable to penetrate and colonize this plant tissues; no symptoms or alteration in gene expression of the major defense signaling pathways were observed. Boava and coworkers (2011) reported the induction of defense-related genes in *Poncirus trifoliata* after *P. parasitica* inoculation and observed the development of small lesions induced by the pathogen. However, the pathogen inoculation by wounding method was used in this experiment (Boava et al. 2011), which should have favored the initial pathogen colonization and triggered a defense response, further impairing disease progression. We assume this proposition because, in natural environmental conditions, *P. parasitica* is unable to surpass structural or chemical barriers to establish an effective colonization. The inoculation method via zoospores herein performed might be a closer mimic to natural interacting conditions and, additionally, no mechanical or wound lesions were supplement to *P. parasitica* inoculation.

The *P. parasitica*–citrus pathosystem represents a hemibiotrophic plant–pathogen interaction. We demonstrated that SA, JA, and ET signaling pathways were activated in *C. sunki* upon infection. Attard et al. (2010) found similar results in *A. thaliana* plants and suggested that these phytohormone pathways co-operate for defense during root infection. We observed that the gene expression levels in *C. sunki* culminated in HR, which is a type of PCD. The HR was confirmed by histochemistry with detection of high levels of peroxidase activity in *C. sunki*–infected tissues (Fig. 7). ROS generation, especially H_2O_2 , have been reported as the first plant-cell response against pathogen attack (Mellersh et al. 2002; Shetty et al. 2003; Thordal-Christensen et al. 1997). During stress conditions, plant cells are able to induce the so-called oxidative burst consisting mainly of H_2O_2 production. This ROS accumulation plays a central role during HR, triggering defense responses. HR has been reported in different plant species, in response to interaction with bacteria, fungi, viruses, oomycetes, nematodes, insects, and parasitic plants (Govrin and Levine 2000; Höglund et al. 2005; Huang et al. 2010; Runyon et al. 2010; Vleeshouwers et al. 2000; Wang and Wang 2016). Our analysis of H_2O_2 accumulation by DAB supports the occurrence of HR in *C. sunki*, but this defense strategy was not sufficient to impair the pathogen colonization. The hemibiotrophic lifestyle of *P. parasitica* allows feeding on dead tissue, and therefore, the HR mechanism employed by *C. sunki* is not efficient, since cell death is induced, which favors the exponential growth of *P. parasitica* in

Fig. 10. Hierarchical clustering of expression of *Phytophthora parasitica* effector genes in interaction with *Poncirus trifoliata* at 0, 3, 6, 12, 24, 48, and 96 h postinoculation (hpi). The heat map illustrates fold change in expression ($\log_2$ scale). Different shades represent induced or repressed gene expression. Apoplastic effectors (PpNPP1.1, PpNPP1.3, PpNPP1.4, PpELI2, PpELI3, PpCBEL1, PpCBEL2, PpCBEL3, PpCBEL4) and cytoplasmic effectors (PpRXL1, PpRXL17, PpCRN4, PpCRN8) were evaluated.

infected root tissues (Figs. 2 and 3). On the other hand, a HR response in *Poncirus trifoliata* plants was not observed (Fig. 8), indicating that *Poncirus trifoliata* resistance is not via HR and is likely to be driven by preformed physical and biochemical barriers found in this citrus species.

The reactions observed in both plant hosts upon infection seem to be an all-or-none response, in which the SA, JA, and ET signaling pathways were insufficient to restrain the development of the pathogen in *C. sunki* plants, while no genetic reprogramming related to these phytohormones was observed in *Poncirus trifoliata*. Additionally, we could not detect any indication of pathogen development in *Poncirus trifoliata*, which suggests that *Poncirus trifoliata* is a nonhost plant species to *P. parasitica*. Several studies have reported that haustoria development fails in nonhost plants (Bettgenhaeuser et al. 2014; Cheng et al. 2012; Ellis 2006). Furthermore, nonhost resistance in *Poncirus trifoliata* might be associated with multiple quantitative trait loci (QTL), as indicated by studies with filamentous pathogens, assuming that the pathogen can secrete distinct effector proteins (Stam et al. 2014).

In *C. sunki*, the candidate effector genes *PpELI2*, *PpELI3*, *PpNPP1.4*, *PpCBEL2*, *PpCBEL4*, and *PpRxLR17* showed upregulation at the beginning of the infection (3, 6, and 12 hpi) (Fig. 9). Elicitins and NPP1 are associated with induction of necrosis (Du et al. 2015; Fellbrich et al. 2002). *CBEL* genes are associated with pathogen adhesion in plant cell surfaces (Gaulin et al. 2002). *RxLR* genes are associated with either defense suppression or activation via manipulation of hormonal pathways (Anderson et al. 2015). Although a functional characterization of these genes is required, the putative secretion of these effectors at the beginning of infection might suggest an induction of necrosis, rapid adhesion and colonization, and hormone signaling disturbance. Altogether, these events would explain the disease progression triggered by *P. parasitica* in *C. sunki* plants.

In *Poncirus trifoliata*, all putative pathogenic effectors had their expression strongly upregulated at the early timepoints

(Fig. 10); nevertheless, this had no effect in transcriptional changes of defense-related genes and other physiological genes. The overall increase of gene transcription that encodes putative effectors, suggests an increasing attempt of *P. parasitica* to break *Poncirus trifoliata* barriers and establish an infective process. Particularly at 6 hpi, genes such as *CBEL*, *ELI*, *RxLR*, and *CRN* were strongly expressed, without induction of tissue necrosis, pathogen colonization, or host immunity manipulation, singly or in combination. It is plausible that *Poncirus trifoliata* seedlings lack one or more molecular targets of those effectors or that it is not necessary for this resistant plant to activate defenses, since there is basically no penetration of hyphae.

The main events recorded for *P. parasitica* interaction along with *C. sunki* and *Poncirus trifoliata* seedlings is summarized in Figure 11. In *C. sunki* plants, this pathogenic oomycete invades root tissues and deploys several molecular effectors already at early stages of infection. The susceptible plant responds to pathogen attack by a strong activation of genes related to the main defense signaling pathways, i.e., SA, JA, and ET, and this major transcriptional reprogram results in HR. However, this is not efficient to kill or prevent pathogen colonization. Because *P. parasitica* has a hemibiotrophic lifestyle, it might benefit from cell death triggered by HR and release of nutrients from the plant. In *Poncirus trifoliata*, otherwise, despite contact with several *P. parasitica* effectors, activation of SA, JA, or ET signaling pathways is not detected. The only physiological shift triggered by *P. parasitica* in this resistant plant was a decrease in water consumption, which indicates that the main defense strategy employed by *Poncirus trifoliata* is its preformed biochemical or anatomical barriers, or both. Future works should take advantage of these data to generate efficient control strategies or resistant plants, based on i) conventional breeding programs, via use of QTL or expressed QTL to generate citrandarins and ii) biotechnology-based approaches, which include the development of transgenic or *cisgenic* citrus, genome editing, and host-induced gene silencing (HIGS).

Fig. 11. Model of the interaction between *Phytophthora parasitica* and citrus plants. **A**, In *Citrus sunki*, *P. parasitica* colonizes root tissues and triggers the expression of several defense-related genes. High reactive oxygen species production and necrosis are observed. Cell death might not be effective as defense strategy against this hemibiotrophic pathogen and may, instead, favor pathogen development. **B**, In *Poncirus trifoliata*, *P. parasitica* colonization is not observed. In this interaction, most defense-related genes had a constitutive expression throughout infection. Expression of apoplastic and cytoplasmic effectors, which may suppress the defense response, is detected. These results indicate that *Poncirus trifoliata* may recognize the pathogen, but its resistance relies mainly on preformed biochemical and anatomical barriers.

MATERIALS AND METHODS

Oomycete growth conditions.

Phytophthora parasitica (isolate IAC_01/95.1) was grown in a carrot-agar medium, at 25°C in the dark. Sporangia development was performed according to Dalio et al. (2014). Briefly, after the colony reaching up to 80% of the plate capacity, sporangia development was induced by pouring sterile water on the plate and replacing water daily for 1 week. To induce release of zoospores, plates were incubated at 4°C, in the dark, for 1 h. Zoospore suspension was collected and was adjusted to a concentration of 10^5 zoospores per milliliter. The number of zoospores in suspension was recorded using a Neubauer chamber.

Plant growth conditions and *P. parasitica* infection assay.

Seeds of *C. sunki* and *Poncirus trifoliata* were germinated in sterile substrate. After 3 months, substrate was carefully removed and plants were transferred to a tube containing 50 ml of sterile distilled water. Zoospore suspension was added to the tube, which was next sealed with parafilm, to avoid water loss to evaporation. Plants were incubated in growth chambers at 20°C, under a 12 h photoperiod ($250 \mu\text{mol m}^{-2} \text{s}^{-1}$ photosynthetic photon flux density). Six biological replicates were used in each treatment and the infection assay was performed in two independent replicates. Daily, plants were monitored for root necrosis, growth of visible mycelia on root surface, wilting leaves, mortality, and water uptake. Plants were also harvested at 0, 3, 6, 12, 24, 48, and 96 hpi and were used to measure *P. parasitica* genomic DNA content and tissue (roots and leaves) damage and for expression analysis of genes from citrus plants and *P. parasitica*.

Microscopy analysis.

Root tips were sampled 96 hpi and were fixed in Karnovsky solution (2% paraformaldehyde, 2.5% glutaraldehyde, and 0.1 M phosphate buffer) for 3 days. Subsequently, they were washed in phosphate buffer, were embedded in paraffin, and were sectioned longitudinally. Each 1 μm section was transferred to slides, stained with 0.05% toluidine blue for 8 min, was analyzed under light microscope, and was photo documented.

ROS measurement in *P. parasitica*-infected plants.

To detect ROS generation (specifically H_2O_2 production) in roots of *C. sunki* and *Poncirus trifoliata* seedlings (24-hpi infected and noninfected plants), we performed a DAB assay according to Thordal-Christensen et al. (1997) and Salzer et al. (1999). Briefly, DAB polymerizes when in contact with H_2O_2 and the peroxidase enzyme, resulting locally in a brownish to black color in the tissues. Stained root samples were subsequently analyzed under a stereoscope light microscopy.

Isolation and quantification of *P. parasitica* genomic DNA.

First, 100 mg of fresh mycelia were ground in liquid nitrogen and the resulting powder was transferred to a 1.5-ml sterile tube. Genomic DNA isolation was then performed using DNeasy plant mini kit (Qiagen, Hilden, Germany), and further purification was performed using the Wizard DNA clean up system (Promega, Mannheim, Germany), according to manufacturer's recommendations. DNA quality and purity were checked on Nanodrop (NanoDrop 8000, Thermo Fisher Scientific, Waltham, MA, U.S.A.).

To quantify *P. parasitica* DNA content, reverse transcription RT-qPCR analyses was performed targeting the *PN5b-PN6* genes. DNA was diluted in H_2O (1:10) to prevent PCR inhibition. RT-qPCR were performed in Applied Biosystems 7500 Real-Time PCR System using GoTaq Real-Time qPCR (Promega), according to manufacturer's recommendations. Briefly, each 20- μl reaction contained 1 μl of DNA template, 1.1 μl of H_2O ,

10 μl of Go-Taq qPCR Master mix, and 0.25 μl of 10-pmol/ μl primer solution. Primers were β -actin PN5b (5' GAACAATG CAACTTATTGGACGTT 3') and PN6 (5' AACCGAAGCTGCC ACCCTAC 3'), generating an amplicon with a fragment size of 120 bp (Ippolito et al. 2002). Amplification conditions were 95°C for 10 min and 40 cycles of 95°C for 15 s, 62°C for 60 s. Melt curve analysis was performed by raising the temperature from 65 to 95°C, in 0.5°C steps for 5 s each, to detect specificity by dissociation of amplicons and primer dimers formation. Reactions were performed in three biological and technical replicates. Cycle threshold values of samples were compared with a standard curve (obtained using genomic DNA isolated from *P. parasitica* grown in petri plates with carrot medium) to determine the concentration of each sample. The standard curve concentrations ranged from 1 pg of DNA ml^{-1} to 10 ng of DNA ml^{-1} in five steps.

Gene expression analysis.

For *C. sunki* and *Poncirus trifoliata* gene-expression analysis, we selected genes related to defense pathways, including SA, ET, and JA, normal physiological conditions, and other general defense-related genes. *Arabidopsis thaliana* gene sequences were obtained from The Arabidopsis Information Resource (TAIR) and were used for a blast search of citrus orthologous genes by BLASTX on Phytozome, selecting the sequence with the highest hit score, based on identity and query cover, i.e., lowest E-value. Primers were designed using Primer3 Plus software, considering the following parameters: 40 to 60% GC content, 18 to 24 nt primer length, annealing temperature of $60^\circ\text{C} \pm 2$, and 70 to 200 bp amplicon length. Total RNA was extracted from 50 mg of root tissue, using MasterPure plant RNA purification kit (Epicentre Biotechnologies, Madison, WI, U.S.A.), followed by DNaseI (Thermo Fisher Scientific) treatment, according to manufacturer's recommendations.

For *P. parasitica* analysis, we developed a bioinformatic pipeline for the prediction of candidate secreted effector proteins, considering the following parameters: presence of signal peptides, predicted by Signalp v.3.0 software (Bendtsen et al. 2004); absence of transmembrane helices and glycosylphosphatidylinositol (GPI) anchors, predicted, respectively, by TMHMM software (Krogh et al. 2001) and GPIsom software (Fankhauser and Mäser 2005). Based on predictions, candidate effectors were retrieved from 22,113 evaluated sequences. Among the predicted sequences, we selected sequences of apoplastic and cytoplasmic effectors (RxLR and CRN effectors) and sequences with predicted domains of known elicitors, such as NPP1 and CBEL. RT-qPCR primers were designed using Primer3 Plus following the same parameters set for citrus, described previously.

Total mRNAs (from *C. sunki*, *Poncirus trifoliata*, and *P. parasitica*) were reverse transcribed using oligo-dT primers in a 20- μl reaction volume, using RevertAid first-strand cDNA synthesis kit (Thermo Fisher Scientific) and 1 μg of total DNA-free RNA. cDNA was diluted (1:20), and 1 μl of the diluted cDNA was used in a 13- μl volume reaction containing 6.75 μl of Go-Taq qPCR Master mix and 0.75 μM (1 μl) of each primer. RT-qPCR were performed in the Applied Biosystem 7500, in triplicate reactions with the following conditions: 95°C for 20 s and 40 cycles of 95°C for 3 s, 60°C for 30 s. Melt curve analysis was performed by raising the temperature from 65 to 95°C, in 0.5°C steps for 5 s each, to detect specificity by dissociation of amplicons and primer dimers formation. A list of primers and sequences used in this study can be found in Supplementary Table S1.

Analysis of housekeeping reference genes.

We tested several housekeeping genes for the normalization of gene expression in *P. parasitica*-infected plants. The

stability of *GAPC2* (glyceraldehyde-3-phosphate dehydrogenase C2), *EGIDH* (NADP-isocitrate dehydrogenase), *DIM1* (DIM1 homolog/YLS8), *CYP* (cyclophilin), *FBOX* (FBOX family protein), *GAPC2* (glyceraldehyde-3-phosphate dehydrogenase C2) was investigated using geNorm and NormFinder software programs. Sequences of these housekeeping genes were searched and retrieved from the databases CitEST and HarVEST.

Statistical analysis.

Differences between the amount of *P. parasitica* genomic DNA in *C. sunki* and *Poncirus trifoliata* roots were analyzed using a linear regression approach, presented in Figure 3. Lines reflect the logarithm base 2 of the amount of genomic DNA (micrograms of DNA per gram) per time course point (3, 6, 12, 24, 48, and 96 hpi) for both plants and the regression statistical significance is reflected in F-test *P* value.

Water uptake rate, in *C. sunki* and *Poncirus trifoliata*, was evaluated at 24, 48, 72, and 96 hpi, using a linear regression model with time course and a dummy explanatory variable to identify control and infected plants.

For statistical analysis of gene expression, quantification cycle value (Cq), and primer efficiency values were calculated from raw fluorescent data (Rn values), using the Real-time PCR Miner program (Zhao and Fernald 2005). Housekeeping genes used for normalization in citrus were *CYP*, *FBOX1*, and *GAPC2*, and, for *P. parasitica*, *UBC* (ubiquitin C) and *S3A* (ribosomal protein S3A) genes. Relative quantification (Rq) was calculated using $2^{-\Delta\Delta Cq}$, and the significance of the results was tested using the Kruskal-Wallis test.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

The *Arabidopsis* Information Resource (TAIR) website:

<http://www.arabidopsis.org>

Phytozome portal: <https://phytozome.jgi.doe.gov/pz/portal.html>

Primer3 Plus software:

<http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi>

Real-time PCR Miner program: <http://ewindup.info/miner/>