# Resistance to citrus canker induced by a variant of *Xanthomonas citri* ssp. *citri* is associated with a hypersensitive cell death response involving autophagy-associated vacuolar processes

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## SUMMARY

*Xanthomonas citri* ssp. *citri (X. citri)* is the causal agent of Asiatic citrus canker, a disease that seriously affects most commercially important *Citrus* species worldwide. We have previously identified a natural variant, *X. citri* $A^T$, that triggers a host-specific defense response in *Citrus limon*. However, the mechanisms involved in this canker disease resistance are unknown. In this work, the defense response induced by *X. citri* $A^T$ was assessed by transcriptomic, physiological and ultrastructural analyses and the effects on bacterial biofilm formation were monitored in parallel. We show that *X. citri* $A^T$ triggers a hypersensitive response associated with the interference on biofilm development and arrest of bacterial growth in *C. limon.* This plant response involves an extensive transcriptional reprogramming setting in motion cell wall reinforcement, oxidative burst and accumulation of salicylic acid (SA) and phenolic compounds. Ultrastructural analyses revealed subcellular changes involving the activation of autophagy-associated vacuolar processes. Our findings show the activation of SA-dependent defense in response to *X. citri* $A^T$ and suggest a coordinated regulation between SA and flavonoids pathways, which is associated with autophagy mechanisms that control pathogen invasion in *C. limon.* Furthermore, this defense response protects *C. limon* plants from disease upon subsequent challenges by pathogenic *X. citri*. This knowledge will allow to rationally exploit the plant immune system as a biotechnological approach to manage the disease.

## INTRODUCTION

*Xanthomonas citri* ssp. *citri* (*X. citri*) strain A is the causative agent of Asiatic citrus canker, a disease that seriously affects most commercially important *Citrus* spp. worldwide (Vojnov *et al.*, 2010). In South America, other phylogenetically different canker-causing *Xanthomonas* were identified, belonging to *X. fuscans* ssp. *aurantifolii* (*X. aurantifolii*) strains B and C (Schaad *et al.*, 2005, 2006). However, *X. aurantifolii* B strain could not be isolated from field after Asiatic citrus canker became endemic in 2002 (Chiesa *et al.*, 2013); and *X. aurantifolii* C strain has a host range restricted to Mexican lime (*Citrus aurantifolia*) in some citrus-producing areas in Brazil (Graham *et al.*, 2004). Therefore, B and C strains are not a serious threat in the field.

*X. citri* is a hemibiotrophic pathogen that grows and persists as epiphytes, forming biofilms on the host surface prior to endophytic colonization of the intercellular spaces of the mesophyll tissue through natural openings, such as stomata, or through wounds (Rigano *et al.*, 2007). A balance between biofilm formation and bacterial dispersion is essential for enhancing epiphytic persistence of bacteria prior to colonization and for circumventing the plant defense response (Favaro *et al.*, 2014; Vojnov and Marano, 2015).

The host defense response is composed of complex and highly-regulated molecular networks, which can be triggered by the perception of either conserved pathogen-associated molecular patterns (PAMPs) or race-specific pathogen effectors (Jones and Dangl, 2006; Macho and Zipfel, 2015). In *Citrus* spp., the first level of defense triggered by *X. citri* has been associated with early molecular changes in gene-expression, particularly linked to the production of reactive oxygen species (ROS) (Cernadas *et al.*, 2008; Enrique *et al.*, 2011). However, in most cases, *X. citri*

disrupts PAMP-triggered immunity (PTI) and produces the disease. In the last decade, different molecular and genetic approaches, including comparative genomics and mutants, have been followed to identify *X. citri* virulence factors or effectors involved in the suppression of PTI leading to canker development. Recently, we have shown that xanthan, the major exopolysaccharide secreted by *Xanthomonas* spp., promotes *C. limon* susceptibility to *X. citri* by suppressing hydrogen peroxide ($H_2O_2$) accumulation (Enrique *et al.*, 2011). The *pthA4* gene encoding type III-secreted transcriptional activator-like (TAL) effector is other well-known pathogenicity effector of canker-causing *Xanthomonas* that contributes to host susceptibility (Duan *et al.*, 1999; Shiotani *et al.*, 2007). Deletion of *pthA4* gene was shown to reduce the bacterial population and abolish the ability of the pathogen to cause canker disease (Domingues *et al.*, 2010; Jia *et al.*, 2015).

The second level of plant immunity is triggered in many plant-pathogen interactions when specific effectors secreted by the pathogen can be recognized by plant resistance (R) proteins, activating the effector-triggered immunity (ETI) (Jones and Dangl, 2006). However, no *R* gene has been identified in citrus yet. Several types of citrus and closely related genera, including 'Chinese' citron (*C. medica)*, calamondin (*C. mitis* Blanco), Yuzu (*C. ichangensis* x *C. reticulata* var. *austera*) and 'Nagami' kumquat (*Fortunella margarita*), have been reported to be fully resistant to *X. citri*, suggesting a specific recognition of avirulence effectors (Chen *et al.*, 2012; Deng *et al.*, 2010; Khalaf *et al.*, 2011; Lee *et al.*, 2009). In this regard, transcriptional responses to *X. citri* in 'Nagami' kumquat include the induction of defense-related genes, particularly those implicated in hypersensitive response (HR) associated with rapid programmed cell death (PCD), a process that restricts the spread of the pathogen and prevent disease development (Khalaf *et al.*, 2011). In Arabidopsis, HR-PCD

induced by avirulent (hemi)biotroph pathogens is associated with the activation of autophagy, an intracellular membrane trafficking pathway with substantial roles both in promotion as in control of vacuole-mediated cell death (Teh and Hofius, 2014). Nevertheless, in canker-resistant genotypes, the mechanisms underlying HR-PCD remains obscure.

*X. citri* natural variants with restricted host range have been isolated worldwide. Two of these variants, named $A^*$ and $A^w$, have a host restricted to *C. aurantifolia* and *C. macrophylla* and induce HR-like reactions in *C. paradisi* and *C. sinensis* (Sun *et al.,* 2004; Vernière *et al.,* 1998). This HR-like phenotype was correlated with the presence of the *xopAG* (syn. *avrGf1*) effector gene, identified in all $A^w$ strains and in three $A^*$ strains (Escalon *et al.,* 2013; Rybak *et al.,* 2009). However, the signalling pathways involved in these HR-like responses remain to be elucidated.

Recently, we have characterized a new variant of *X. citri*, named $A^T$, which shares more than 90% genetic similarity with the type A pathogenic strain *X. citri* T. Despite this high similarity, the host range of this variant is restricted to *C. aurantifolia* and *C. clementina.* In *C. limon,* this strain triggers an atypical chlorotic phenotype associated with a host-specific defense response (Chiesa *et al.,* 2013).

In this work, we assess the molecular and cellular events underlying the response of *C. limon* to *X. citri* $A^T$. We show that this variant triggers a HR-PCD associated with the interference on biofilm development and the activation of autophagy-related vacuolar processes. The defense response involves cell wall reinforcement, accumulation of phenolic compounds and induction of salicylic acid (SA) signaling pathway. Moreover, pre-inoculation with *X. citri* $A^T$ confers resistance to the pathogenic strain *X. citri* T.

## RESULTS

### Biofilm formation is impaired and bacterial growth is arrested in the *C. limon*-*X. citri* $A^T$ interaction

Proper biofilm formation is a requirement to achieve maximal *X. citri* virulence (Malamud *et al.,* 2013) and the ability of canker-resistant *Citrus* spp. to interfere with this process has been reported in 'Okitsu' mandarin (Favaro *et al.*, 2014). In this work, we examined whether the impaired ability of *X. citri* $A^T$ to cause disease in *C. limon* was associated with its inability to develop biofilms. Interestingly, no significant differences were observed between both *X. citri* $A^T$ and the pathogenic *X.citri* T strain, neither in the initial adhesion (1 to 3 h) nor in biofilm development (15 to 24 h) to polystyrene microplates (Fig. S1, see Supporting Information). Next, green fluorescent protein (GFP)-tagged *X. citri* strains were inoculated in young *C. limon* leaves and the ability of *X. citri* $A^T$ to develop biofilms and bacterial growth was monitored. Up to 2 days post-inoculation (dpi), epiphytic growth of both *X. citri* strains was similar on *C. limon* leaves (data not shown). At 7 dpi, biofilm formation was seen only with *X. citri* T and not with *X citri* $A^T$ (Fig. 1a). By contrast, both strains could develop biofilms on *C. clementina* leaves (Fig. 1a). Here, both bacterial aggregates showed a three-dimensional structure on ZX-axis projected images with formation of compact microcolonies (Fig. 1a). These are similar structures to those reported previously for *X. citri* biofilm formed on the susceptible genotypes *C. limon* and *C. clementina* (Favaro *et al*., 2014; Rigano *et al.,* 2007). Moreover, inoculation of both strains onto *C. clementina* leaves led to the development of cankerous lesions after 20 dpi (data not shown). Nevertheless, *X. citri* $A^T$ and T elicited different macroscopic symptoms in *C. limon* leaves at 20 dpi; *X. citr*i $A^T$ induced discrete black

spots, phenotypically different to the canker lesions caused by *X. citri* T (Fig. 1b). Trypan blue staining revealed that *X. citri* $A^T$ induced cell death response at 48 hpi in *C. limon*, while no cell death was observed after inoculation of *X. citri* T over the monitored period (Fig. 1c).

Taken together, these results indicate that *X. citri* $A^T$ is able to form microcolonies and develop biofilms on both non-biotic and certain biotic surfaces, and suggest that it is the induction of defense responses specifically in *C. limon* that interferes with biofilm development and the arrest of bacterial growth.

**A distinct set of *C. limon* genes mediates canker resistance**

Transcriptome analysis was performed to gain insight into the molecular mechanisms mediating the cell death phenotype observed in *C. limon* plants inoculated with *X. citri* $A^T$. Leaves were inoculated with bacterial suspensions of both *X. citri* strains and samples were harvested at 48 hours post-inoculation (hpi). Differential gene expression analysis identified 1079 up-regulated and 1832 down-regulated genes in the interaction with *X. citri* $A^T$ (fold change $\geq 2$ in inoculated vs. non-treated plants, false discovery rate (FDR) $\leq 5\%$). A lower but substantial number of genes were differentially expressed in the compatible *C. limon*-*X. citri* T interaction (869 and 1036, up- and down-regulated, respectively) (Table S2, see Supporting Information). Comparison of both transcriptomic responses revealed that an important number of genes were specifically expressed only by one of the two bacteria (Fig. S2, see Supporting Information). Particularly, 1455 genes (461 and 994 up- and down-regulated, respectively) were unique to *X. citri* $A^T$ response (Table S3, see Supporting Information). Functional analysis identified 104 gene ontology (GO) categories statistically enriched in the *X. citri* $A^T$ interaction and 62 in the *X. citri* T

interaction. Again, as observed at the transcript level, we could glimpse a number of biological processes that were distinct between both interactions (Table S4, S5, see Supporting Information). Some of these processes are highlighted in Table 1.

These data indicate that both strains trigger an important rearrangement of *C. limon* transcriptome and although some of these responses are shared by the two interactions, there are distinct responses that are exclusively triggered by *X. citri* $A^T$. Some of these processes were studied in detail using a combination of molecular, physiological and ultrastructural analyses.

**Defense response to *X. citri* $A^T$ is associated with cell wall reinforcement and accumulation of phenolic compounds**

Different genes related to cell wall modification were regulated in response to both *X. citri* strains, likely influencing the final outcome of each interaction. In *C. limon*, *X. citri* $A^T$ down-regulates genes like xyloglucan:xyloglucosyl transferase, *XTH6* and *XTH16* (2.8-fold repressed), involved in cell wall loosening; cellulose synthases, *CESA7* and *CSLC12* (3.8- and 3-fold repressed, respectively); pectinesterases, such as *PME3* and *SKS6* (4.1 and 3.8-fold repressed), and β-1,3 glucanases (3.3-fold repressed), suggesting an active reinforcement of the plant cell wall through the increase of pectin methyl esterification and callose deposition (Table S3). Conversely, *X. citri* T up-regulates expansin genes, such as *βEXP2* and *EXPA4* (64 and 2.7-fold induced, respectively), which promote the weakening of the plant cell wall, resulting in cell enlargement (hypertrophy) and division (hyperplasia) required for canker development (Cernadas *et al.*, 2008; Fu *et al.*, 2012) (Table S3). *βEXP2* expression was also analyzed by qRT-PCR, confirming the microarray results (Table S6, see Supporting Information). Functional analysis using GO also revealed

that categories such as 'cell wall thickening' ($3.4x10^{-2}$ adjusted *P*-value) and 'defense response by callose deposition in cell wall' ($2.5x10^{-2}$ adjusted *P*-value) were enriched upon the up-regulated genes by *X. citri* $A^T$ (Table 1). On the other side, the category 'cell wall modification involved in multidimensional cell growth' ($2.8x10^{-2}$ adjusted *P*-value) was enriched in the *C. limon*-*X. citri* T interaction (Table 1). Taken together, these results suggest that *C. limon* response to *X. citri* $A^T$ is associated with a fortification of the cell wall, limiting growth and spread of the bacteria.

The categories 'flavonoid biosynthetic process' ($3.6x10^{-4}$ adjusted *P*-value) and 'glucosinolate metabolic process' ($1.6x10^{-2}$ adjusted *P*-value) were enriched upon genes up-regulated by *X. citri* $A^T$, suggesting that the biosynthesis of these secondary metabolites is fostered in this interaction (Table 1). Particularly, two key genes involved in indolic glucosinolates (GSL) biosynthesis, cytochrome P45083B1 (*CYP83B1*) and UDP-glucosyl transferase74B1 (*UGT74B1*) (7.6-fold and 2.6-fold induced, respectively), were exclusively up-regulated against *X. citri* $A^T$ (Table S3). Although the differential expression of these two genes was confirmed (Table S6), we were not able to detect GSL in these samples (Roeschlin *et al.*, unpublished), indicating either that their concentration is below our detection limit, or more likely that these genes are indeed participating in another metabolic pathway. In addition, genes involved in flavonoids and anthocyanins biosynthesis such as phenylalanine ammonie lyase (*PAL1)* (2.9-fold induced), chalcone synthase (*CHS1*) (4-fold induced), flavanol 3-hydroxylase (*F3H*) (2-fold induced), flavonol 3´-hydroxylase (F3´H) (3-fold induced), downy mildew resistant 6 (*DMR6*) (5.5-fold induced), and anthocyanidin-3-O-glucosyltransferase (*3GT*) (2.3-fold induced) were all up-regulated only in response to *X. citri* $A^T$ (Table S3). The same trend was observed by qRT-PCR for *PAL1* and *CHS1*, as observed in Figure 2a. Confirming these results, histological

assays showed the accumulation of bright green fluorescent polyphenolic compounds, particularly in the abaxial side and around stomata, in *X. citri* $A^T$-inoculated leaves at 48 hpi (Fig. 2b). Notably, this accumulation was higher at 7 dpi. By contrast, leaves inoculated with *X. citri* T did not show accumulation of phenolic compounds, as indicated by the homogeneous red fluorescence along the tissue, generated by the autofluorescence of chlorophyll (Fig. 2b). Moreover, spectrophotometric determinations confirm that the content of flavonoids and anthocyanins increased significantly in response to *X. citri* $A^T$, supporting the idea that phenolic compounds are implicated in this host-specific defense response (Fig. 2c).

**_X. citri_ $A^T$ down-regulates genes related to ROS scavenging and photosynthesis**

The production of ROS is one of the earliest cellular responses following successful pathogen recognition. Apoplastic generation of superoxide ($O_2^-$), or its dismutation product $H_2O_2$, can cause strengthening of the plant cell walls mediating signaling for gene activation and promoting HR-PCD (Chi *et al.*, 2013). It was previously observed that the *C. limon-X. citri* $A^T$ interaction led to an increased production of $H_2O_2$ (Chiesa *et al.*, 2013), suggesting the deployment of a *bona fide* defense response leading to canker resistance. Several ROS-related genes regulated in response to *X. citri* $A^T$-inoculation were found in this work, indicating that the redox homeostasis has been altered in the plant cell (Table S2). Microarray data indicate that a respiratory burst NADPH-oxidase homolog to the Arabidopsis *RBOHD*, the main enzyme responsible for the oxidative burst upon pathogen infection (Kadota *et al.*, 2014), was induced 5.2-fold, a result that was confirmed by qRT-PCR (Table S2, S6). In addition, copper/zinc superoxide dismutase (*SOD2*) and its chaperone (*CCS*), increased their expression 2.3-fold, whereas ROS scavengers like catalases (*CAT3*) or

peroxidases (*PER64*, *PER68*) were down regulated (Table S3). A different redox response was observed in leaves inoculated with the pathogenic *X. citri* T. Although the expression of *RBOHD* gene was also slightly up-regulated, this induction was two-fold lower than in the *X. citri* $A^T$ interaction (Table S2). Moreover, 'Hydrogen peroxide catabolic process' was enriched ($1.8x10^{-4}$ adjusted *P*-value) (Table 1).

Additionally, the GO category related to 'photosynthesis' was enriched upon the down-regulated genes in response to *X. citri* $A^T$ ($1.9x10^{-4}$ adjusted *P*-value, Table 1). This category includes genes encoding thylakoid proteins such as light-harvesting complex, *LHCB6* and *LCHB1.4*; components of the oxygen-evolving complex of photosystem II, *PSBO-1* and *PSBO-2*; and genes involved in the Calvin cycle, *RBCS1A*, *RBCS2B*, *FBA1* and *RCA* (Table S3). This rapid down-regulation of photosynthesis may also be associated with the high level of ROS production shown in the *C. limon-X. citri* $A^T$ interaction, as it was demonstrated in ETI responses (Liu *et al.*, 2007; Shapiguzov *et al.*, 2012).

**SA is involved in the local defense response induced by *X. citri* $A^T$**

SA is thought to act with ROS in a feed-forward loop, promoting HR-PCD, as demonstrated in defense responses against (hemi)biotrophic pathogen infections (Mammarella *et al.*, 2014; Wrzaczek *et al.*, 2013). Interestingly, functional analysis identified the GO category 'response to salicylic acid' enriched upon the up-regulated genes unique to the *C. limon-X. citri* $A^T$ interaction ($2.4x10^{-3}$ adjusted *P*-value, Table 1). Belonging to this category, genes involved in SA biosynthesis, signaling and response were up-regulated. For instance, as described before, the expression of *PAL1* was induced 2.9-fold, and genes involved in the biosynthesis of methylsalycilate, such as S-adenosylmethionine-dependent methyltransferases (*SAMT* and *BSMT1)*, were

induced 2.4 and 4.4-fold, respectively. The same tendency was found for the key regulator of SA signaling nonexpressor of pathogenesis-related genes1 (*NPR1*, 2.6-fold), the transcription factor *WRKY70* (4.4-fold) and the pathogenesis-related (*PR*) genes *PR1* (32.7-fold) and *PR4* (14.1-fold) (Table S3). The induction of the first three key genes of the SA pathway was confirmed by qRT-PCR (Fig. 3a).

SA quantification showed that its concentration increased 3-fold in *X. citri* $A^T$-inoculated leaves at 48 hpi, compared to control samples (Fig. 3b). This significant rise was not observed at 7 dpi, time point in which biofilm development begin to decline (Fig. 1a), suggesting a temporal regulation of SA signaling in the defense response against *X. citri* $A^T$. By contrast, no differences in SA levels were observed between *X. citri* T-inoculated leaves and control samples (Fig. 3b).

**Subcellular analysis suggest autophagy-mediated vacuolar cell death events in *X citri* $A^T$-inoculated *C. limon* leaves**

In this work, it was shown that *X. citri* $A^T$ triggers a host-specific defense response associated with HR-PCD. To further characterize the subcellular changes induced by *X. citri* $A^T$, samples from bacteria-inoculated leaves were analyzed by TEM. Immediately after inoculation of *C. limon* (0 hpi), tissues did not present any cellular change (Fig. 4a,g). At 48 hpi, *X. citri* T-inoculated samples showed the presence of bacteria colonizing the leaf surface (Fig. 4b) and invading the mesophyll cells (Fig. 4c). At 7dpi, bacteria were present within the damaged mesophyll cells (Fig. 4d), and they became more abundant in the intercellular space at 20 dpi (Fig. 4e), when canker symptoms are already visible (Fig. 4f). As it was expected, in *C. clementina* similar ultrastructural changes were triggered either by *X. citri* T or *X. citri* $A^T$, consistent with the ability of both bacteria to cause the disease (Fig. S3).

316 By contrast, although *X. citri* $A^T$-inoculated *C. limon* leaves showed dispersed
317 bacteria on the leaf surface (Fig. 4h) and in the intercellular space (Fig. 4i), the
318 epidermal pavement cells resemble empty and tightly cemented at 48 hpi, suggesting
319 cellular collapse that is characteristic of HR-vacuolar cell death (Hatsugai *et al.*, 2009;
320 Rojo *et al.*, 2004; Van doorn *et al.*, 2011). Associated with these processes, higher
321 magnification images showed bacteria with irregular cell shape, undergoing
322 degenerative processes (Fig. 4i). Furthermore, mesophyll cells showed vacuole
323 membrane invaginations, suggesting loss of vacuole turgor (Fig. 4j), and a perforated
324 nuclear envelope wrapped by tubular extensions (Fig. 4k), all features of vacuolar cell
325 death (Van doorn *et al.*, 2011).
326 Next, at 7 dpi collapsed mesophyll cells were observed, suggesting the rupture
327 of the tonoplast and the release of the vacuolar content (Fig. 4l). On the other hand,
328 mesophyll cells with intact chloroplasts showed the presence of autophagosome-like
329 vesicles (Fig. 4m). The formation of autophagosomes (double membrane vesicles) is a
330 hallmark of the activation of authophagy-mediated pathway (Van doorn *et al.*, 2011).
331 At 20 dpi, MET analysis exhibit similar results to those obtained before, showing two
332 types of mesophyll cell responses. While some of them were dead, with thickening of
333 the cell wall and accumulation of electron-dense multitextured materials filling the
334 intercellular space (Fig. 4n), others showed signs of chloroplast enlargement (Fig. 4o)
335 and an increase in the number and size of autophagosomes (Fig. 4p,q), suggesting that
336 an active autophagy-regulated mechanism in surviving cells is involved in restriction
337 of the spreading of the HR-PCD.
338 Taken together, these results suggest that *X. citri* $A^T$ induces a HR-PCD in *C.*
339 *limon*, mediated by vacuolar cell death associated with autophagy. At early times post
340 inoculation, these autophagic processes would prevent bacterial colonization through

vacuolar cell death, but later on, it may restrict the spreading of the cell death process itself.

### *X. citri* $A^T$ protects *C. limon* from canker development

In order to investigate whether the host response triggered by *X. citri* $A^T$ is able to induce plant protection to the pathogenic strain *X. citri* T, young *C. limon* leaves were pre-inoculated by cotton swab with bacterial suspensions of *X. citri* $A^T$-GFP. Forty-eight hpi, the leaves were challenged with *X. citri* T-GFP by spraying (Fig. 5a). A significant reduction in canker development was observed in leaves pre-inoculated with *X. citri* $A^T$-GFP as compared to mock-inoculated leaves (Fig. 5b). Similar results were obtained when pre-inoculation with *X. citri* $A^T$-GFP was performed by spraying (data not shown). In a new assay, both bacteria were co-inoculated to *C. limon* in equal amounts. Under these conditions, cankerous lesions were observed (Fig. 5c), discarding bacterial competition being the determinant of the *X. citri* $A^T$-induced protection observed before.

These data suggest that *X. citri* $A^T$ triggers a defense response that protects *C. limon* from canker disease.

## DISCUSSION

### *X. citri* $A^T$ triggers a recognition event interfering with biofilm development in *C. limon*

In this work we demonstrate that *X. citri* $A^T$ is able to develop biofilms on *C. clementina* and cause disease. The presence of *X. citri* $A^T$ inside the damaged *C. clementina* mesophyll cells implies the ability of these bacteria to dissolve the host cell wall and disrupt the cell, inducing similar morphological changes that pathogenic *X. citri* T. These ultrastructural modifications during citrus canker development have been well reported in *X. citri*-inoculated Mexican lime samples (Lee *et al.*, 2009). On the other hand, although *X. citri* $A^T$ is able to colonize the leaf surface and the intercellular spaces of the mesophyll tissue, it fails to develop a mature biofilm structure in *C. limon*. The presence of degenerated *X. citri* $A^T$ bacteria near the cell wall might be linked to the release of vacuolar hydrolytic enzymes during the HR-PCD response, affecting the biofilm development. Altogether, these results indicate that bacterial biofilm formation constitutes not only a virulence factor of canker-causing *Xanthomonas*, but also its disruption could be used as a marker of canker resistance response.

### *X. citri* $A^T$ triggers a HR-PCD response which is associated with elevated levels of flavonoids and SA

The phenotypes triggered by *X. citri* strains in *C. limon* are associated with an extensive transcriptional reprogramming. An important degree of commonality between both interactions with different outcome is found, which may be related to the similar genetic backgrounds of the two strains (Chiesa *et al.*, 2013). Therefore,

this common subset of genes could be accounting for PTI basal response, as was previously reported between *C. sinensis*-*X. aurantifolii* C/*X. citri* interactions (Cernadas *et al.*, 2008). The most striking differences are observed in the number of unique genes regulated during *X. citri* $A^T$ infection when compared with the response to pathogenic *X. citri* T. From the total of unique genes considered differentially-expressed, nearly 76% correspond to *X. citri* $A^T$-triggered response. In a similar way, *X. aurantifolii* C induces a greater number of defense-related genes than *X. citri* infection in *C. sinensis*, suggesting that the amplitude of this response is sufficient to halt *X. aurantifolii* C growth and establish an effective HR (Cernadas *et al.*, 2008). In contrast, a relatively small number of defense-related genes were up-regulated in the partially resistant 'Meiwa' kumquat cultivar to *X. citri*, when compare with susceptible *C. sinensis* (Fu *et al.*, 2012). Overall, our results are consistent with the contention that the *C. limon* defense response to *X. citri* $A^T$ is governed by the recognition of bacterial effectors. Another remarkable feature of the resistance response is that the number of repressed genes is doubled as compared to the susceptible response (pathogenic interaction). The down regulation of genes coding for development and photosynthesis proteins coupled with the up regulation of genes coding for defense proteins, points to a possible cross-talk between these biological processes in the infected tissue. This regulation would allow a better management of the energy resources, as was previously reported in other interactions that are known to be ETI-mediated (Bilgin *et al.*, 2010; Karpinsky *et al.*, 2013).

The maintenance of host cell wall integrity in response to *X. citri* $A^T$, through the repression of the xyloglucan-cellulose network and the production of highly methyl esterified pectins, may protect it from bacterial enzymatic degradation. The fact that the increase in PME activity leads to enhanced *Pseudomonas syringae*

susceptibility in Arabidopsis reinforces this idea (Bethke *et al*, 2014). Interestingly, *X. aurantifolii* C also down-regulates the *XTHs* genes in *C. sinensis* (Cernadas *et al.*, 2008), suggesting that this repression plays a protective role in the defense response, limiting pathogen invasion. Furthermore, thickening of the cell wall by increased callose deposition is the first barrier not only to *X. citri* infection in citrus plant (Enrique *et al.*, 2011), but also in other plant-bacterial interactions (Hauck *et al.*, 2003; Yun *et al*., 2006; Voigt, 2014). In agreement with these results, the repression of *β-1,3 glucanase* and the reinforcement of cell wall were observed in response to *X. citri* $A^T$, which are correlated with the beginning of cell death and the restriction of bacterial colonization in *C. limon*.

The repression of cellulose biosynthesis genes and the lignin biosynthetic pathways, may lead to the accumulation of secondary metabolites. In Arabidopsis, cellulose synthase (*CESA*7)-deficient mutants increased the resistance to broad range of pathogens, through the up-regulation of defense-related genes, including those involved in the accumulation of antimicrobial secondary metabolites (Hernandez-Blanco *et al.*, 2007). Moreover, Vanholme *et al.* (2012) proposed that a reduced flow of the lignin biosynthesis pathway may lead to a higher availability of substrates for the biosynthesis of phenolic compounds. In our work, GO analysis reveals that while the category of 'lignin biosynthetic process' is not significantly represented, several categories related to phenylpropanoid pathways are enriched upon the up-regulated genes by *X. citri* $A^T$. According with this, at early times post inoculation an increase of antimicrobial phenylpropanoids, including flavonoids and anthocyanins is observed. Phenolic deposits have also been reported around the HR lesions triggered by *X. citri* in the 'Nagami' kumquat and calamondin resistant plants (Chen *et al.*, 2012).

Over recent years, significant progress has been made to understand the role of SA in regulating plant defense response to pathogen attack (Fu and Dong, 2013; Kazan and Lyons, 2014). However, there is no data on the activation of SA-dependent defense in response to *X. citri* in citrus. Here, we show an accumulation of SA at early time of *X. citri* $A^T$ inoculation, which is correlated with the beginning of the HR-PCD in *C. limon*. Interestingly, at later stages of defense response, while SA decreases to basal levels, the accumulation of phenolic compounds continues to rise. In Arabidopsis and maize, it has been proposed that flavones act as signal molecules modulating the SA levels under abiotic and biotic stress conditions (Falcone *et al.*, 2015; Pourcel *et al.*, 2013). Our results, show a temporal regulation of SA in *X. citri* $A^T$ resistance response, and suggest a coordinated regulation between SA and flavonoids pathways.

**HR triggered by *X. citri* $A^T$ involves autophagy-associated vacuolar processes protecting the plant from canker development**

Autophagy has emerged as a central process in the regulation of pathogen-triggered HR. In the last years, several studies in model plants have shown that defense-related autophagy is involved both in cell survival (pro-survival; avoiding spread of HR) and cell death (pro-death) (Hofius *et al.*, 2011; Seay and Dinesh-Kumar, 2005; Teh and Hofius, 2014; Zhou *et al.*, 2014). However, the mechanism governing this molecular switch is not well understood. In this work, we provide different lines of evidence that *X. citri* $A^T$ triggers an ETI-like response in *C. limon*, where the execution and inhibition of HR-PCD is associated with autophagic processes, temporary regulated. In the last years, it has been shown that SA signals play an important role in the induction of autophagy, which in turn operates as a

458 negative regulator of SA-dependent signaling, restricting the spread of HR-PCD. In
459 Arabidopsis, autophagy-related genes (*ATG)*-mutants have shown an increase of SA
460 levels leading to the ETI-associated spreading of PCD during *P. syringae* effector
461 AvrRPM1 challenge. These results suggest that autophagy is a critical mechanism to
462 control the HR-mediated PCD (Liu *et al.,* 2005; Xia *et al.,* 2013; Yoshimoto *et al.,*
463 2009). According to our results, the formation of autophagosome-like vesicles in
464 survival cells and the reduction of the SA level at 7 dpi suggest that autophagy-
465 associated vacuolar processes also may regulate the cell death spreading.

466 In Arabidopsis, the vacuole-mediated PCD triggered by the *P. syringae*
467 effector AvrRPM1 was associated with two different pathways, the proteasome-
468 regulated membrane fusion and the activation of the vacuolar processing enzyme
469 (VPE)-dependent defenses (Hatsugai *et al.,* 2009; Rojo *et al.,* 2004). In this regard,
470 during the HR-PCD triggered by *X. citri* $A^T$, GO analysis showed that the category
471 'catabolic protein process', which involves proteasome activity, was not significantly
472 represented in response to *X. citri* $A^T$. However, we observed that vacuole-mediated
473 cell death goes along with the up-regulation of γ-*VPE* gene that is associated with the
474 formation of double membrane autophagosome-like vesicles and the up-regulation of
475 *ATG8f*. Accumulation of transcripts of *ATG8* gene family has been reported in
476 pathogen infected Arabidopsis plants and they are widely used to monitor temporal
477 regulation and subcellular dynamics of autophagy processes (Hofius *et al.,* 2011;
478 Kabbage *et al.,* 2013; Yoshimoto *et al.,* 2004). Consequently, our results suggest that
479 *X. citri* $A^T$-triggered HR is mediated by a vacuolar-membrane collapse that releases
480 the antimicrobial content into the cytoplasm, causing the cell death. Although, in *X.*
481 *citri* $A^T$ we were not able to detect the presence of the *xopAG* effector gene (data not

482 shown), other bacterial effectors should be involved in triggering the HR-PCD in *C.*
483 *limon*.

484 To our knowledge, this is the first report of the molecular mechanisms
485 involved in HR induction by *X. citri* variants in commercially important citrus
486 species, setting our results as a novel study to exploit the plant immune system as a
487 biotechnological approach to manage the disease. Moreover the fact that pre-
488 inoculation with *X. citri* $A^T$ confers resistance to the pathogenic *X. citri*, establish the
489 bases for an eventual biological control of citrus canker.

## EXPERIMENTAL PROCEDURES

### Plant material, bacterial strains, and pathogenicity assays

One year-old 'Eureka' lemon (*C. limon* (L.) Burm. f.) plants grafted onto Troyer citrange and 'Clemenules' mandarin (*C. clementina* Hort. ex Tan.) grafted onto *Poncirus trifoliata*, were kept under controlled conditions in a growth chamber. New shoots approximately 1 cm long, with at least five leaves, were selected for pathogenicity assays after pruning the plants. All the leaves on a new shoot were considered to be of the same ontological age (Favaro *et al.*, 2014).

*X. citri* strains were transformed by electroporation with plasmid pMP2444 expressing GFP (Rigano *et al.,* 2007). Bacterial suspensions ($10^9$ cfu/mL) were prepared in 10 mM $MgCl_2$ and inoculated by spraying or cotton swab on 15-day-old leaves of the new shoots. A 10 mM $MgCl_2$ solution was used as mock inoculation. Inoculated plants were maintained for 30 days in a growth chamber as previously reported (Enrique *et al.*, 2011). Disease progression was phenotypically monitored using a stereomicroscope MVX10 and photographed under white and UV light (520 nm). The canker lesions were quantified per square centimeter, using Image J software (v1.41; National Institutes of Health, Bethesda, MD, USA).

Images in Figures 1, 2, 4 and 5 are representative results from three independent biological replicates each one involving three different plants and three different leaves per plant.

### Biofilm analysis

Bacterial adhesion and biofilm formation *in vitro* were performed as previously described (Rigano *et al.,* 2007).

Biofilm formation *in vivo* was examined using GFP-tagged *X. citri* strains and an inverted confocal laser-scanning microscope as described previously (Favaro *et al.*, 2014). Simulated three-dimensional images and sections were generated by the software Nikon EZ-C1 3.9 Free Viewer.

**Histochemical and transmission electron microscopy (TEM) assays**

Cell death was visualized in *C. limon* leaves after staining with lactophenol–trypan blue, as previously described (Koch and Slusarenko, 1990). Autofluorescence of phenolic compounds was observed by fluorescence microscopy (excitation at 450-490 nm, emission at 520 nm) (Chen *et al.*, 2012) by using free-hand leaf sections (Lux *et al.*, 2005). Observations were performed with an Olympus BX50F4 microscope

For TEM experiments, leaf pieces (2x3 mm) were fixed in 4% (v/v) glutaraldehyde in phosphate buffer (1.8 g/L $NaH_2PO_4$; 23.25 g/L $Na_2HPO_4.7H_2O$ and 5 g/L NaCl, pH 7.4) for 24 h at 4°C, and processed according to standard protocols. Sections were examined with a TEM (JEOL-100CXII, Tokyo, Japan) at an accelerating voltage of 80 kV and digital images were recorded with a ES1000W CCD digital camera (Gatan Inc., CA, USA).

**RNA Preparation**

Total RNA from *C. limon* leaves (4 g) were grinded in liquid nitrogen and homogenized in 15 mL extraction buffer (200 mM Tris-HCl pH 8.5; 200 mM sucrose; 30 mM magnesium acetate; 60 mM KCl; 0.5% (w/v) polyvinylpyrrolidone; 0.5% (w/v) sodium deoxicolate; 1% (w/v) SDS; 1% (w/v) sodium-*n*-lauroylsarcosine; 10 mM EDTA; 2% (v/v) β-mercaptoethanol). Extraction procedure was performed as

previously described (Marano and Carrillo, 1992). RNA samples were purified over Qiagen RNeasy mini-columns (Hilden, Germany).

**Microarray experiments**

Five inoculated leaves were randomly harvested at 48 hpi from three different plants and considered as an independent biological replicate. Three biological replicates were done.

RNA samples were amplified using the Amino Allyl MessageAmp$^{TM}$ II aRNA amplification kit (Applied Biosystems, Van Allen Way Carlsbad, CA, USA). Reverse transcription, cDNA purification, dye coupling and fluorescent cDNA purification were performed according to manufacturer´s instructions. A citrus microarray developed by the Interdisciplinary Center for Biotechnology Research (ICBR) of the University of Florida, and Agilent Technologies Inc (Palo Alto, CA, USA) was used. This microarray contains 44000 probes based on citrus expressed sequence tags (ESTs) from *Rutaceae* (Febres *et al.*, 2012). Microarray hibridization was performed according to the manufacturer´s instructions (Agilent Gene Expression Hybriditation kit, Agilent Technologies, Palo Alto, CA, USA). The slides were scanned with GenePix Pro 4000B and analyzed with GenePix6.0 software (Axon instruments, Sunnyvale, CA, USA). Those features with background-subtracted intensity lower than two-fold the local background intensity in the two channels were discarded. Raw data were normalized as described in Martinez-Godoy *et al.* (2008). Only features with valid data in the three replicates were considered for further analysis.

**Microarray data analysis**

564 The identification of differentially-expressed genes was performed using
565 significance analysis of microarrays test (SAM) (Tusher *et al*, 2001). A 5% FDR and
566 2-fold expression cut off were considered to determine up- and down-regulated genes.
567 Functional analysis was carried out using FatiGO (Babelomics 4.0, Medina *et al.*,
568 2010), considering statistically significant those GO terms having an adjusted *P*-
569 value≤ 0.05. Microarray data have been deposited in the Gene Expression Omnibus
570 (GEO) database under the accession number GSE78013.
571
572 **Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)**
573 The qRT-PCR reactions were performed according to Enrique *et al.*, (2011).
574 Reactions were carried out with real-time PCR master mix (Biodynamics SRL, BA,
575 Argentina) and monitored in the Mastercycler® ep realplex system (Eppendorf,
576 Hamburg, Germany). Primers used in the experiments are listed in Table S1
577 (Supporting Information). Transcript levels were normalized against histone H4
578 (Shiotani *et al.*, 2007) using the ΔΔCt method (Livak and Schmittgen, 2001). Non-
579 treated (NT) lemon leaves served as the reference sample.
580
581 **Quantification of UV-absorbing compounds and SA**
582 Spectrophotometric determination of phenolic compounds, flavonoids and
583 anthocyanins, were performed according to Mazza *et al.* (2000) and Falcone *et al.*
584 (2010), respectively.
585 For SA quantification, leaves samples were obtained as described for
586 microarray experiments. SA was extracted from 200 mg freeze-dried leaves according
587 to a previously described method (Durgbanshi *et al.*, 2005). Samples were injected
588 into a liquid chromatographer (LC) coupled with electrospray tandem mass

589 spectrometry (MS/MS, Quattro Ultima, Micromass, MAN, UK). Masslynx NT
590 version 4.0 (Micromass) software was used to process the chromatograms.

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**SUPPORTING INFORMATION LEGENDS:**

**Figure S1. Bacterial adhesion and biofilm formation on inert plastic surface.**

**Figure S2. Venn diagrams representing the distribution of regulated transcripts in *Citrus limon* after *Xanthomonas citri* ssp. *citri* (*X. citri*) inoculation.**

**Figure S3. Transmission electron microscopy of *Citrus clementina* leaves inoculated with *Xanthomonas citri* ssp. *citri* (*X. citri*) strains.**

**Table S1. List of all oligonucleotide primers used for quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis.**

**Table S2. Microarray expression data for up- and down-regulated genes in response to *Xanthomonas citri* ssp. *citri* (*X. citri*) strains in *Citrus limon* leaves.**

**Table S3. Comparison of microarray expression data for up- and down-regulated genes in *Citrus limon* leaves in response to *Xanthomonas citri* ssp. *citri* (*X. citri*) strains.**

**Table S4. Gene ontology (GO) 'biological process' terms enriched in the differentially-expressed genes unique to *C. limon* - *Xanthomonas citri* ssp. *citri* strain $A^T$ interaction.**

868 **Table S5. Gene ontology (GO) 'biological process' terms enriched in the**
869 **differentially-expressed genes unique to *C. limon* - *Xanthomonas citri* ssp. *citri***
870 **strain T interaction.**

871

872 **Table S6. Quantitative reverse transcription-polymerase chain reaction (qRT-**
873 **PCR) analysis of genes involved in defense and pathogenesis response to**
874 ***Xanthomonas citri* ssp. *citri* (*X. citri*) strains.**

875

## FIGURE LEGENDS

**Figure 1. Host-specific response triggered by *Xanthomonas citri* ssp. *citri* (*X. citri*) strain $A^T$. (a)** Biofilm formation on *Citrus limon* and *C. clementina* leaves at 7 days post-inoculation (dpi). Red chlorophyll fluorescence and green signals from green fluorescent protein (GFP)-tagged *X. citri* strains are shown. XY and ZX are the XY and ZX axis projected images, respectively. Scale bar, 50 µm. **(b)** Macroscopic symptoms in *C. limon* leaves at 20 dpi. Leaves were photographed under white and UV light. Scale bar, 10 mm. **(c)** Microscopic cell death phenotype (arrows) observed at 48 hours post-inoculation. Scale bar, 150 µm.

**Figure 2. Phenolic compounds are involved in *Citrus limon* response to *Xanthomonas citri* ssp. *citri* (*X. citri*) strain $A^T$. (a)** Quantitative reverse transcription-polymerase chain reaction analysis of phenylalanine ammonie lyase (*PAL1)* and chalcone synthase (*CHS1)* mRNAs were measured at 48 hours post-inoculation (hpi). Relative gene expression (ΔΔCt) fold change of mRNA levels was performed considering non-treated plants as reference sample and histone H4 transcript as an endogenous control. Values are expressed as means±SD from three independent biological replicates. The data set marked with an asterisk is significantly different as assessed by Tukey's test ($P< 0.05$). **(b)** Light microscopic images of lemon leaves inoculated with *X. citri* strains. Leaves were photographed at 48 h and 7 days post-inoculation (dpi) under white and UV light. Green fluorescent polyphenol compounds (arrows) and red chlorophyll fluorescence are observed. Scale bar, 10 µm. **(c)** Spectrophotometric determination of flavonoids and anthocyanins at 48 hpi. Values are expressed as means±SD. Each sample consists in 10 leaf discs (0.5-cm

diameter) obtained from two shoots of three different plants and ten biological replicates were performed. The data set marked with an asterisk is significantly different as assessed by Tukey's test ($P< 0.05$). A, absorbance.

**Figure 3. *Xanthomonas citri* ssp. *citri* (*X. citri*) strain $A^T$ triggers accumulation of salicylic acid (SA) in *Citrus limon*. (a)** Quantitative reverse transcription-polymerase chain reaction analysis of *NPR1* (nonexpressor of pathogenesis-related genes 1), *WRKY70* transcription factor and pathogenesis-related (*PR1*) mRNAs were measured at 48 hours post-inoculation (hpi). Relative gene expression (ΔΔCt) fold change of mRNA levels was performed considering non-treated plants (NT) as reference sample and histone H4 transcript as an endogenous control. Values are expressed as means±SD from three independent biological replicates. The data set marked with an asterisk is significantly different as assessed by Tukey's test ($P< 0.05$). **(b)** Analysis of SA through LC-MS/MS performed at 48 h and 7 days post-inoculation (dpi). Values are expressed as means±SD from three independent biological replicates. The data set marked with an asterisk is significantly different as assessed by Tukey's test ($P< 0.05$). DW, dry weight tissue.

**Figure 4. Ultrastructural features of *Citrus limo*n leaves inoculated with *Xanthomonas citri* ssp. *citri* (*X. citri*) strains. (a, g)** At 0 hours post-inoculation (hpi) nucleus, vacuole and chloroplast are intact. **(b)** Bacteria are localized on the leaf surface and **(c)** within the mesophyll cells. **(d)** Bacteria colonizing mesophyll tissue. **(e)** Bacteria in the intercellular space. Arrows, electron-dense multitextured materials. **(f)** Breakdown of epidermal tissue and canker formation. **(h)** Bacteria colonizing the leaf surface and **(i)** the intercellular spaces. Arrows, epidermal tissue collapse. Upper

926 panel shows the magnification of degenerated bacteria. **(j)** Arrows, vacuole
927 membrane invaginations. **(k)** Arrow, perforations of nuclear envelope. **(l)** Arrows,
928 cellular collapse. **(m)** Arrows, autophagosomes-like vesicles. **(n)** Cell death and
929 accumulation of electron-dense multitextured materials. **(o-q)** Arrows,
930 autophagosomes-like vesicles. Scale bar, 2 µm. b, bacteria; C, canker; CD, cell death;
931 ch, chloroplast; cw, cell wall; db, degenerated bacteria; ep, epidermis; is, intercellular
932 space; ls, leaf surface; n, nucleus; sg, starch granules; t, tubular extensions; v, vacuole.
933
934 **Figure 5. Pre-inoculation with *Xanthomonas citri* ssp. *citri* (*X. citri*) strain $A^T$**
935 **protects *Citrus limon* to canker disease. (a)** Phenotypic response of lemon leaves
936 pre-inoculated with *X. citri* $A^T$-tagged with green fluorescent protein (GFP) or mock-
937 inoculated by cotton swab. Forty-eight hours post-inoculation, the leaves were
938 subsequently challenged, via spraying, with the pathogenic *X. citri* T-GFP strain.
939 Sections from the left panels are shown magnified on the right panels. Leaves were
940 photographed under white and UV light. Scale bar, 10 mm. **(b)** Number of canker
941 lesions per square centimeter in pre-inoculated leaves at 20 days post-inoculation
942 (dpi). Values are expressed as means±SD from three independent biological replicates
943 each one involving three different plants and five different leaves per plant. The data
944 set marked with an asterisk is significantly different as assessed by Student's t test
945 ($P< 0.05$). **(c)** Canker symptoms developed at 20 dpi of lemon leaves co-inoculated
946 with equal amounts of both bacteria strains by cotton swab. Scale bar, 10 mm.