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Plant responses underlying nonhost resistance of *Citrus limon* against *Xanthomonas campestris* pv. *campestris*

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SUMMARY

Citrus is an economically important fruit crop that is severely afflicted by citrus canker, a disease caused by *Xanthomonas citri* ssp. *citri* (*X. citri*); thus, new sustainable strategies to manage this disease are needed. Although all *Citrus* spp. are susceptible to this pathogen, they are resistant to other *Xanthomonas* species, exhibiting non-host resistance (NHR), for example, to the brassica pathogen *X. campestris* pv. *campestris* (*Xcc*) and a gene-for-gene host defence response (HDR) to the canker-causing *X. fuscans* ssp. *aurantifolii* (*Xfa*) strain C. Here, we examine the plant factors associated with the NHR of *C. limon* to *Xcc*. We show that *Xcc* induced asymptomatic type I NHR, allowing the bacterium to survive in a stationary phase in the non-host tissue. In *C. limon*, this NHR shared some similarities with HDR; both defence responses interfered with biofilm formation, and were associated with callose deposition, induction of the salicylic acid (SA) signalling pathway and the repression of abscisic acid (ABA) signalling. However, greater stomatal closure was seen during NHR than during HDR, together with different patterns of accumulation of reactive oxygen species and phenolic compounds and the expression of secondary metabolites. Overall, these differences, independent of *Xcc* type III effector proteins, could contribute to the higher protection elicited against canker development. We propose that *Xcc* may have the potential to steadily activate inducible defence responses. An understanding of these plant responses (and their triggers) may allow the development of a sustained and sustainable resistance to citrus canker.

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INTRODUCTION

Citrus is an economically important fruit tree crop in a world that is severely afflicted by Asiatic citrus canker, a disease caused by the bacterium *Xanthomonas citri* ssp. *citri* (*X. citri*) (Vojnov *et al.*, 2010). Most of the world's citrus species and cultivars are moderately to highly susceptible to *X. citri* and, once established, the pathogen is difficult to eradicate. Quarantine is used to prevent spread to new geographical regions and an integrated disease control programme is applied in endemic regions that may conduct to the destruction of diseased trees and surrounding areas (Canteros *et al.*, 2017; Ference *et al.*, 2018). Thus, there is a great need for new, long-lasting and sustainable strategies to manage citrus canker disease.

Two types of innate immune response against pathogen infections have been described: host and non-host resistance. These are distinguished by whether the pathogen can (host defence response, HDR) or cannot (non-host resistance, NHR) adapt to a particular plant species. Both responses involve, as an initial step, the perception of conserved pathogen-associated molecular patterns (PAMPs) by cell surface pattern recognition receptors (PRRs) (Senthil-Kumar and Mysore, 2013; Tang and Wang, 2017).

NHR is the defence response shown by an entire plant species to all genetic variants of a pathogen (Heath, 2000; Thordal-Christensen, 2003). It is predominantly a multigene trait and

provides more ample and durable resistance against pathogen infection than HDR under field conditions (Senthil-Kumar and Mysore, 2013). NHR may involve the preformed physical and chemical barriers that prevent pathogen ingress, as well as induced defences triggered by PAMPs, referred to as PAMP-triggered immunity (PTI). However, if the recognition involves a pathogen effector that triggers a hypersensitive cell death response (HR), the NHR overlaps with an effector-triggered immunity (ETI) (Adlung *et al.*, 2016; Senthil-Kumar and Mysore, 2013). NHR is classified into type I or type II according to the absence or presence of HR, respectively (Mysore and Ryu, 2004). NHR against bacteria are not fully understood; most studies have been performed in the model plants Arabidopsis and *Nicotiana benthamiana* (Adlung *et al.*, 2016; An and Mou, 2012; An *et al.*, 2017; Ham *et al.*, 2007; Lee *et al.*, 2017; Li W *et al.*, 2012; Li X *et al.*, 2005; Nagaraj *et al.*, 2015). Strong evidence in several pathosystems reveals that induced defences include stomatal immunity, the production of reactive oxygen species (ROS), callose deposition in the cell wall, enhanced expression of defence-related genes and responses to hormones, such as salicylic acid (SA) (An and Mou, 2012; Gill *et al.*, 2015; Lee *et al.*, 2017). A mechanistic understanding of NHR provides great promise for the development of sustainable broad-spectrum resistance in crops, including citrus (Gill *et al.*, 2015; Lee *et al.*, 2017). This goal might be achieved through the identification and subsequent manipulation of defence or signalling components associated with NHR.

In *Citrus sinensis*, a type II NHR has been shown against *X. campestris* pv. *vesicatoria* (*Xcv*) (Daurelio *et al.*, 2013), a pepper and tomato pathogen (Thieme *et al.*, 2005). Transcriptomic analysis indicates that this NHR modulates genes associated with biotic stress, cell death and plant hormone signalling (Daurelio *et al.*, 2013, 2015; Petrocelli *et al.*, 2018). In contrast, the mechanistic basis of type I NHR in *Citrus* ssp. remains unknown. Previously, we have provided evidence that the interaction of *C. paradisi* and *C. limon* with *X. campestris* pv. *campestris* (*Xcc*) is asymptomatic (Chiesa *et al.*, 2013), suggesting that *Citrus* spp. are non-hosts of this bacterium. *Xcc* is the major bacterial pathogen of plants of the Brassicaceae family, including Arabidopsis, and is an economically important phytopathogen of cruciferous plants worldwide (Vicente and Holub, 2013). In Arabidopsis, *Xcc* can enter into the leaf through hydathodes, wounds and stomata (Cerutti *et al.*, 2017; Gudesblat *et al.*, 2009). Similarly to *X. citri*, *Xcc* requires a functional type III secretion (T3S) system to deliver effector proteins into their host cells and to cause disease. However, the *Xcc* genome does not contain the pathogenicity effector *pthA4* gene (da Silva *et al.*, 2002), which contributes to canker development (Duan *et al.*, 1999).

HDR is often considered as a gene-for-gene response and is usually very specific in a particular plant genotype or cultivar and against a particular pathogen genotype (Dodds and Rathjen, 2010; Zipfel, 2014). *Xanthomonas fuscans* ssp. *aurantifolii* (*Xfa*) strain C triggers HDR in most *Citrus* spp., except in *C. aurantifolia* in which it induces canker disease. This response is associated with an HR at the site of bacterial infection that limits pathogen growth (Brunings and Gabriel, 2003; Chiesa *et al.*, 2013; Gochez *et al.*, 2015). Recently, an *Xfa* effector (AvrGf2/XopAG) has been identified as responsible for triggering the HR (Gochez *et al.*, 2015, 2017), suggesting that this HDR is predominantly regulated by an ETI. However, as yet, no resistance (*R*) genes have been identified in citrus. In *C. sinensis*, the HDR triggered by *Xfa* is associated with an extensive transcriptional reprogramming, particularly linked to ROS production, cell wall remodelling and hormone synthesis and signalling (Cernadas *et al.*, 2008). However, the physiological and biochemical responses triggered by *Xfa* in *Citrus* spp. at the beginning of the interaction are still unknown.

In this work, we have analysed the early physiological, biochemical and molecular responses of *C. limon* associated with the NHR triggered by *Xcc*, and compared these with the responses associated with the HDR triggered by *Xfa.* Our findings indicate that *Xcc* induces a type I NHR in *C. limon*, a response sharing several components with the HDR triggered by *Xfa*. However, differences between the two induced responses occurred in the maintenance of stomatal closure and the accumulation of ROS and secondary metabolites, which could be responsible for the greater protection triggered by *Xcc* against *X. citri* in *C. limon.* In addition, the defence activation is independent of *Xcc* type III effector proteins, suggesting that this NHR is based on PTI.

RESULTS

Type I NHR is induced against *Xcc* in *C. limon*

To investigate the interactions of *C. limon* with *Xcc* and *Xfa,* cultivars 'Eureka' and 'Genova' were inoculated with bacterial suspensions by spraying and pressure infiltration. Green fluorescent protein (*GFP*)-labelled strains of *Xcc* and *Xfa* were used to analyse the phenotype developed over a 16-day post-inoculation (dpi) period. A pathogenic strain of *X. citri* was used as reference for canker development (Roeschlin *et al.*, 2017). The results obtained for 'Eureka' are shown in Fig. 1.

Regardless of the method used to inoculate the bacterium, *Xcc* did not induce macroscopic symptoms in leaves of both cultivars, up to 16 dpi. In contrast, *Xfa* triggered a typical HR, manifested by defined necrotic lesions on sprayed leaves or total necrosis on the infiltrated leaf area after 5 dpi (Fig. 1a). The quantification of bacterial populations *in planta* following inoculation via spraying revealed no significant difference between the strains up to 2 dpi (Fig. 1b). After 2 days, the *Xcc* population remained stable until 8 dpi, whereas *Xfa* population growth began to decline and no bacteria could be recovered at 8 dpi. We hypothesize that this was a consequence of the induction of the HR associated with cell death (Fig. 1a). Similar results were

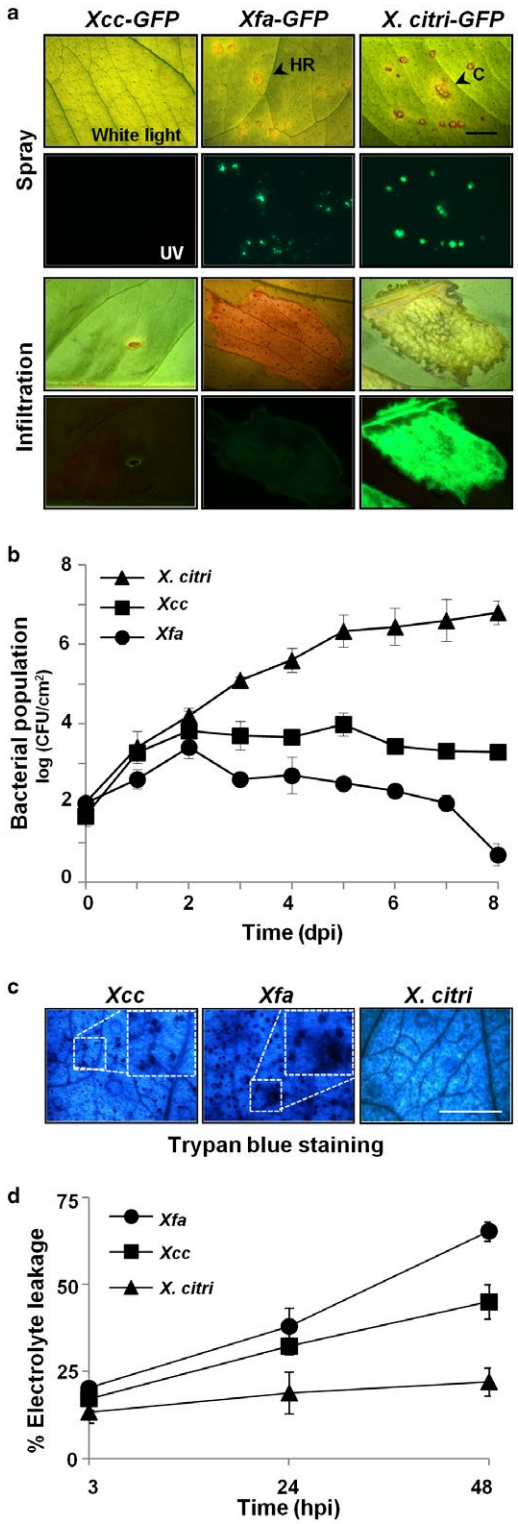

Fig. 1 Phenotypic responses induced by *Xanthomonas* spp. in *Citrus limon*. (a) Macroscopic phenotype on abaxial surface of 'Eureka' lemon leaves at 16 days post-inoculation (dpi) with *Xanthomonas campestris* pv. *campestris* (*Xcc*), *X. fuscans* ssp. *aurantifolii* (*Xfa*) strain C and *X. citri* ssp. *citri* (*X. citri*) by spraying [10^9 colony-forming units (CFU)/mL] and pressure infiltration (10^7 CFU/mL). Leaves were photographed under white and UV light. Arrows: hypersensitive response (HR) and canker (C). Scale bar, 10 mm. (b) Bacterial population growth on *C. limon* leaves inoculated by spraying. Values are expressed as means ± standard deviation (SD) of three independent biological replicates. (c) Microscopic cell death phenotype observed at 48 h post-inoculation (hpi). Insets show the amplification of microscopic cell death. Scale bar, 150 mm. (d) Quantification of cell death in leaves treated as described in (c) by measuring the percentage of electrolyte leakage at 3, 24 and 48 hpi. Values are expressed as means ± SD of three independent biological replicates. [Colour figure can be viewed at wileyonlinelibrary.com]

obtained when the inoculation was performed by pressure infiltration (data not shown).

In addition, trypan blue staining revealed that, although the phenotype developed by *Xcc* in *C. limon* was macroscopically asymptomatic, the bacterium induced a microscopic cell death response at 48 hpi. No cell death was observed after *X. citri* inoculation. Meanwhile, *Xfa* induced a considerable cell death in the inoculated tissue during the evaluated period (Fig. 1c). Quantification of cell death by measurement of electrolyte leakage confirmed this phenotype (Fig. 1d).

Taken together, these results indicate that *Xcc* induces a type I NHR, associated with microscopic cell death, allowing *Xcc* to survive in a stationary phase in *C. limon* tissue.

NHR and HDR disrupt biofilm formation in *C. limon*

Bacterial biofilm formation constitutes a virulence factor of canker-causing xanthomonads and its disruption can be used as a marker of the canker resistance response (Favaro *et al.*, 2014; Roeschlin *et al.*, 2017; Sena-Vélez *et al.*, 2015; Vojnov and Marano, 2015). In addition, biofilm formation is a key factor for *Xcc* pathogenicity (Bianco *et al.*, 2016). We hypothesized that the NHR induced by *Xcc* and the HDR triggered by *Xfa* may contribute to a reduced ability to develop biofilms on *C. limon* leaves. As shown in Fig. 2a, these *Xanthomona*s strains are able to adhere (1 h) and develop biofilm (48 h) on polystyrene microplates. *In planta*, no differences were observed in bacterial adhesion between the three strains at 18 h, with bacteria found particularly at the epidermal cell junctions and around stomata (Fig. 2b). Nevertheless, *Xcc* and *Xfa* failed to form microcolonies or complex structures at 7 dpi, as those developed by *X. citri* with a three-dimensional structure projected on the *ZX* axis (Fig. 2c).

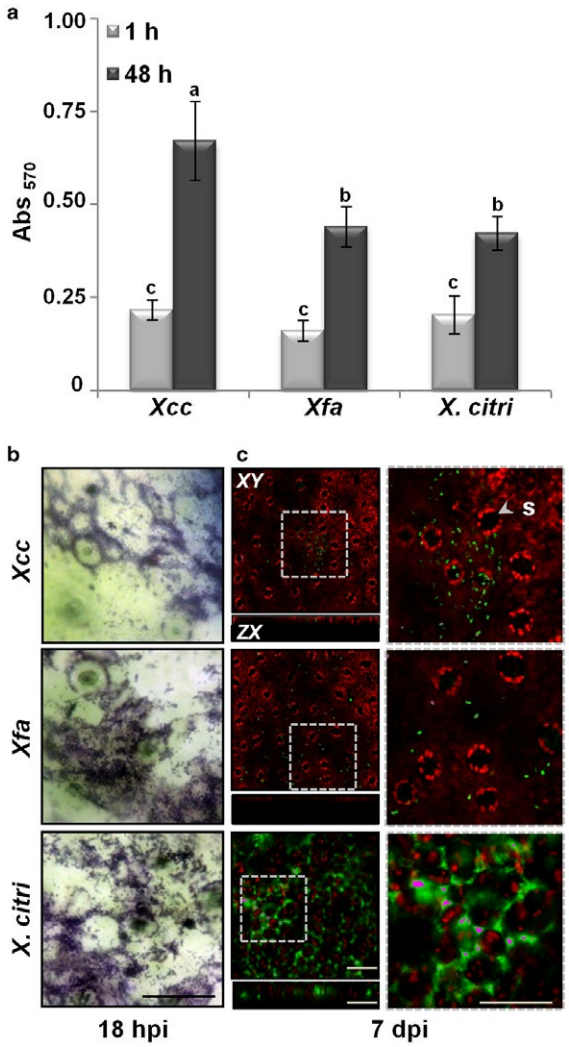

Fig. 2 Non-host resistance (NHR) and the host defence response (HDR) disrupt biofilm formation in *Citrus limon*. (a) Bacterial adhesion and biofilm formation of *Xanthomonas campestris* pv. *campestris* (*Xcc*), *X. fuscans* ssp. *aurantifolii* (*Xfa*) strain C and *X. citri* ssp. *citri* (*X. citri*) on an inert plastic surface after 1 and 48 h of incubation. Values are expressed as means ± standard deviation (SD), $n = 24$. Different letters indicate significant differences at $P < 0.05$ [two-way analysis of variance (ANOVA), Tukey's test]. (b) Bacterial adhesion of *Xcc*, *Xfa* and *X. citri* on abaxial surfaces of *C. limon* leaves assessed by crystal violet staining at 18 h post-inoculation (hpi). Stained cells attached to the leaf surface were analysed microscopically. (c) Biofilm formation of *Xanthomonas* spp. on leaves inoculated by spraying [10^9 colony-forming units (CFU)/mL], observed using confocal laser scanning microscopy at 7 days post-inoculation (dpi). Red indicates chlorophyll fluorescence and green indicates green fluorescent protein (*GFP*)-tagged bacteria. *XY* and *ZX* are the *XY* and *ZX* axis projected images, respectively. Sections from the left panels are shown magnified in the right panels. Arrow: stomata (S). Scale bar, 50 μm. [Colour figure can be viewed at wileyonlinelibrary.com]

These results indicate that the NHR and HDR against *Xcc* and *Xfa*, respectively, interfere with the biofilm development on *C. limon* leaves.

Xanthomonas-triggered stomatal closure is sustained in *C. limon* NHR and HDR

Stomata are the main gateways of foliar pathogenic xanthomonads to gain access into the apoplast for their potential multiplication (Gudesblat *et al.*, 2009; Melotto *et al.*, 2017). In Arabidopsis, the promotion of stomatal closure by pathogenic and non-pathogenic bacteria is mediated by the perception of PAMPs, such as flagellin elicitor peptide (flg22) and lipopolysaccharides (Melotto *et al.*, 2006, 2017; Zeng *et al.*, 2010).

In order to explore the roles of stomatal defence in the NHR and HDR to *Xanthomonas* ssp. in *C. limon*, bacterial promotion of stomatal closure was analysed at 1 and 4 h post-inoculation (hpi). Leaf tissue was inoculated by spraying with *Xcc*, *Xfa* and the pathogenic *X. citri* bacterial suspensions. Abscisic acid (ABA) and water were used as controls of stomatal closure and opening, respectively. Within the first hour of inoculation (1 hpi), the three bacteria promoted stomatal closure when compared with ABA treatment (Fig. 3a). Nevertheless, the average width of the stomatal aperture showed significant differences between the non-pathogenic (*Xcc* and *Xfa*) and pathogenic (*X. citri*) strains (Fig. 3b). At 4 hpi, neither *Xcc* nor *Xfa* could reverse stomatal closure, as happens with *X. citri* to fully colonize its host (Fig. 3b). Moreover, *Xcc* triggered a higher level of stomatal closure than *Xfa*, a response that persisted until 8 hpi (data not shown). These findings suggest that the stomatal-based defence in *C. limon* is stronger during the NHR response to *Xcc* than during the HDR to *Xfa*.

A different pattern of ROS accumulation is observed in NHR and HDR

ROS, including hydrogen peroxide (H_2O_2), superoxide ($O_2^{\bullet-}$) and hydroxyl radical ($^{\bullet}OH$), are important components of multiple signalling pathways triggered by the perception of conserved PAMPs and race-specific pathogen effectors (Macho and Zipfel, 2015). Moreover, ROS are key signal molecules involved in the regulation of stomatal closure (Melotto *et al.*, 2017; Singh *et al.*, 2017; Toum *et al.*, 2016). To gain further insights into the *C. limon* NHR and HDR, ROS accumulation and expression of the $O_2^{\bullet-}$ scavenger, copper/zinc superoxide dismutase (*SOD2*), were analysed. Interestingly, $O_2^{\bullet-}$ accumulation was not observed in tissue inoculated with *Xcc* over the monitoring period (Fig. 4a). Accordingly, *SOD2* was induced 1.5-fold at 3 hpi, increasing to two-fold at 24 hpi, compared with mock treatment (Fig. 4b). These results support the accumulation of H_2O_2 in the NHR against *Xcc* (Fig. 4c). Conversely, in *Xfa*-inoculated leaves, $O_2^{\bullet-}$ accumulation was detected at 3 hpi, compared with mock treatment, diminishing considerably at 24 hpi (Fig. 4a). This $O_2^{\bullet-}$ decrease was associated with the up-regulation of *SOD2* (five-fold) at 24 hpi (Fig. 4b). At the beginning of the two incompatible interactions, H_2O_2 accumulation was shown within the stomatal guard cells

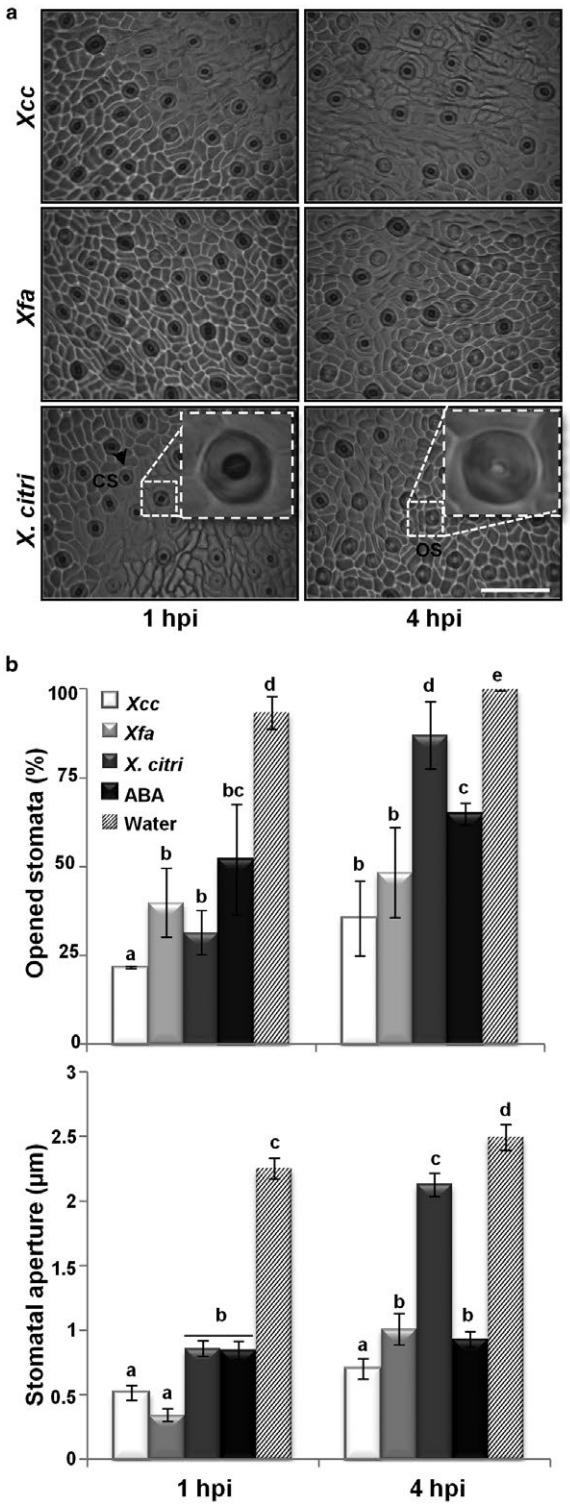

Fig. 3 Non-host resistance (NHR) and the host defence response (HDR) involve the maintenance of stomatal closure in *Citrus limon*. (a) Dried-gel imprint of intact *C. limon* epidermis showing opened (OS) and closed (CS) stomata, shown enlarged in the insets, in leaves inoculated by spraying with bacterial suspension [10^9 colony-forming units (CFU)/mL] of *Xanthomonas campestris* pv. *campestris* (*Xcc*), *X. fuscans* ssp. *aurantifolii* (*Xfa*) strain C and *X. citri* ssp. *citri* (*X. citri*). Scale bar, 100 μm. (b) Quantification of percentage of open stomata and stomatal aperture at 1 and 4 h post-inoculation (hpi) in leaves exposed to *Xcc*, *Xfa*, *X. citri* infection and abscisic acid (ABA) or water treatments by spraying. Values are expressed as the means ± standard deviation (SD) from three independent biological replicates ($n = 60$ stomata). Different letters above the bars indicate significant differences at $P < 0.05$ [two-way analysis of variance (ANOVA), Tukey's test].

(Fig. 4c). These results demonstrate that, although a differential pattern of $O_2^{\bullet-}$ accumulation is elicited in NHR and HDR, H_2O_2 is an important component of both responses in *C. limon*.

NHR involves cell wall reinforcement and the induction of secondary metabolic pathways

We have shown previously that plant cell wall-associated defence is an initial barrier against *Xanthomonas* infection in citrus plants and that callose accumulation is suppressed by *X. citri* during pathogenesis in *C. limon* (Enrique *et al.*, 2011). Moreover, the defence response triggered by canker-forming *Xanthomona*s spp. revealed an extensive transcriptional reprogramming, in which genes involved in cell wall strengthening, and phenylpropanoid and indolic glucosinolate (IGS) biosynthesis, play an important role in resistance (Cernadas *et al.*, 2008; Chen *et al.*, 2012; Roeschlin *et al.*, 2017). Furthermore, IGSs are required for callose deposition, induced by flg22, playing a role as an effective physical barrier at the sites of pathogen attack in Arabidopsis (Clay *et al.*, 2009). In addition, increased levels of IGS are associated with the overexpression of *miR393*, a microRNA (miRNA) that targets auxin receptors and renders Arabidopsis less susceptible to biotrophic pathogens (Robert-Seilaniantz *et al.*, 2011). To further investigate the contribution of these defence markers in *C. limon* NHR to *Xcc*, in comparison with HDR to *Xfa*, the expression of genes involved in the phenylpropanoid pathway (*PAL1*), IGS biosynthesis (*CYP83B1*, *csi-miR393*) and the accumulation of callose and phenolic compounds was analysed.

The relative expression of *PAL1* was increased significantly in response to avirulent bacteria, its expression being 1.6-fold higher in response to *Xcc* than to *Xfa* at 3 and 24 hpi. Meanwhile, the expression of *CYP83B1* triggered by *Xfa* was 18- and 13-fold higher than that triggered by *Xcc* and *X. citri*, respectively, at 3 hpi. Interestingly, at 24 hpi, *CYP83B1* profiles changed and the level was about seven-fold higher in *Xcc*- than in *Xfa*-inoculated leaves (Fig. 5a). Remarkably, at 3 hpi, *csi-miR393* levels were about 1.7-fold higher in *Xcc*- than in *Xfa*-inoculated leaves. At 24 hpi, *csi-miR393* levels in *Xcc*- and *Xfa*-inoculated tissues were reduced significantly; nevertheless, they were two-fold higher than in *X. citri*-inoculated leaves (Fig. 5a). These results suggest

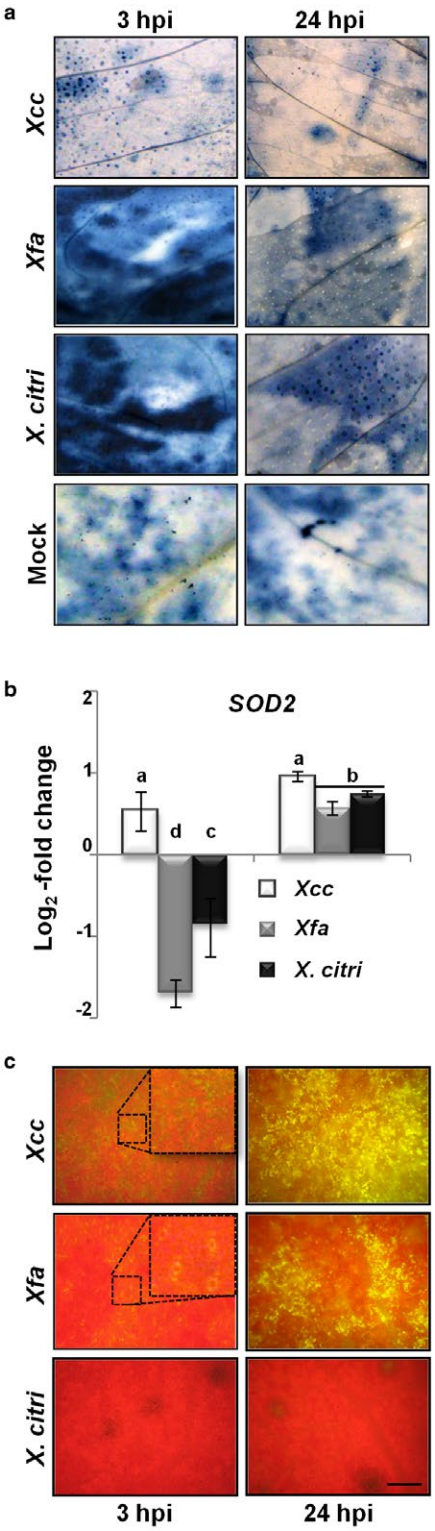

Fig. 4 Different patterns of reactive oxygen species (ROS) accumulation are triggered in non-host resistance (NHR) and the host defence response (HDR). (a) *In situ* accumulation of superoxide radicals ($O_2^{\bullet-}$) detected by dark blue formazan precipitate in *Citrus limon* leaves inoculated by pressure infiltration with bacterial suspensions of *Xanthomonas campestris* pv. *campestris* (*Xcc*), *X. fuscans* ssp. *aurantifolii* (*Xfa*) strain C and *X. citri* ssp. *citri* (*X. citri*), plus mock. (b) Quantitative reverse transcription-polymerase chain reaction analysis of copper/zinc superoxide dismutase (*SOD2*). mRNAs measured at 3 and 24 h post-inoculation (hpi). Relative gene expression ($\Delta\Delta$Ct) fold change of mRNA levels was performed considering mock-treated plants as reference sample and histone *H4* transcript as an endogenous control. Values are expressed as means ± standard deviation (SD) from three independent biological replicates. Different letters indicate significant differences at $P < 0.05$ [two-way analysis of variance (ANOVA), Tukey's test]. (c) H_2O_2 accumulation at 3 and 24 hpi in *C. limon* leaves infiltrated with bacterial suspensions of the different *Xanthomonas* strains [10^7 colony-forming units (CFU)/mL]. Leaves were stained with 2′,7′-dichlorofluorescein diacetate (DCFH-DA) and observed by fluorescence microscopy. H_2O_2 accumulation in the guard cells is shown enlarged in the top insets. Scale bar, 100 μm. [Colour figure can be viewed at wileyonlinelibrary.com]

an association between the induction of *CYP83B1* and *csi-miR393*, and the possible implication of IGS-related pathways, in citrus defence responses, turning on mechanisms of cell wall reinforcement.

Moreover, callose deposition was induced at 24 hpi, as indicated by the number of bright light blue spots per square millimetre in *Xcc*-inoculated (37.5 ± 5.3) and *Xfa*-inoculated (45.3 ± 11.5) tissue (Fig. 5b). No callose deposition was observed in leaves inoculated with *X. citri*. At 7 dpi, the accumulation of bright green fluorescent polyphenolic compounds visible under UV light was observed in *C. limon* leaves inoculated with either of the two non-pathogenic bacterial strains. However, the fluorescence pattern was different between NHR and HDR (Fig. 5c). In NHR against *Xcc*, the autofluorescence was widely spread within the mesophyll tissue. In contrast, in HDR, the autofluorescence was concentrated and co-localized with the HR lesions (Fig. 5c).

NHR and HDR involve the induction of SA-related defence genes and the suppression of ABA signalling in *C. limon*

It has been shown that the SA signalling pathway plays a critical role in the regulation of NHR against *X. citri* in Arabidopsis (An and Mou, 2012). Likewise, ABA has emerged as a multifaceted modulator of the different layers of plant defences, and its role depends on the timing of recognition and the invasive strategy of the challenging pathogen (Asselbergh *et al.*, 2008; Lievens *et al.*, 2017; Petrocelli *et al.*, 2018; Ton *et al.*, 2009). These data prompted us to investigate, through the analysis of the expression of some key genes, whether SA and ABA signalling are also crucial in *C. limon* NHR against *Xcc*.

SA-related gene expression analysis revealed that the key regulator of SA signalling, non-expressor of pathogenesis-related genes 1 (*NPR1*), and the pathogenesis-related 1 (*PR1*) genes were significantly induced by *Xcc* and *Xfa* at 3 and 24 hpi

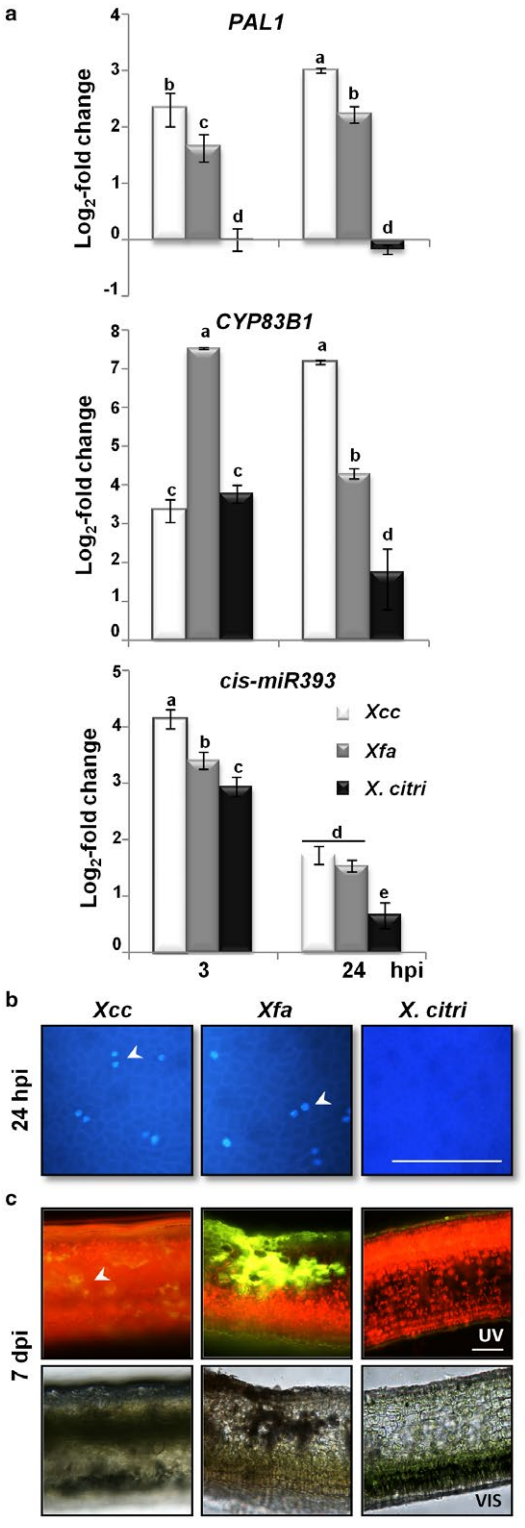

Fig. 5 Cell wall reinforcement through phenolic compounds and callose deposition is involved in *Citrus limon* non-host resistance (NHR) and the host defence response (HDR). (a) Quantitative reverse transcription-polymerase chain reaction analysis of phenylalanine ammonia lyase (*PAL1*), *CYP83B1* and *cis-miR393* in *Citrus limon* leaves inoculated by pressure infiltration with bacterial suspensions [10^7 colony-forming units (CFU)/mL] of *Xanthomonas campestris* pv. *campestris* (*Xcc*), *X. fuscans* ssp. *aurantifolii* (*Xfa*) strain C and *X. citri* ssp. *citri* (*X. citri*). mRNAs were measured at 3 and 24 h post-inoculation (hpi). Relative gene expression ($\Delta\Delta$Ct) fold change of mRNA levels was performed considering mock-treated plants as reference sample and histone *H4* transcript as an endogenous control. Values are expressed as means ± standard deviation (SD) from three independent biological replicates. Different letters indicate significant differences at $P < 0.05$ [two-way analysis of variance (ANOVA), Tukey's test]. (b) Callose deposition in *C. limon* inoculated leaves, stained with aniline blue (arrows). Scale bar, 100 mm. (c) Light microscopic images of *C. limon* inoculated leaves, photographed at 7 days post-inoculation (dpi) under white and UV light. Green fluorescent polyphenolic compounds and red chlorophyll fluorescence are observed. Scale bar, 10 μm. [Colour figure can be viewed at wileyonlinelibrary.com]

(Fig. 6a), although *Xcc*-induced gene expression was observed to a much lesser extent compared with that in *Xfa*-infected tissue. The same tendency was observed with the expression levels of the transcription factor *WRKY70*, suggesting that the SA signalling pathway is induced in the *C. limon* NHR and HDR (Fig. 6a).

With regard to the involvement of ABA in *C. limon* NHR and HDR against non-pathogenic bacteria, we analysed the expression of the *NCED3* (*9-cis-epoxycarotenoid dioxygenase*) gene, which catalyses the rate-limiting step of ABA biosynthesis (Finkelstein, 2013), and the *MYB101* and *csi-miR159* genes, which act as positive and negative regulators, respectively, of the ABA response in Arabidopsis (Curaba *et al.*, 2014; Dubos *et al.*, 2010; Reyes and Chua, 2007). As shown in Fig. 6b, *NCED3*, *csi-miR159* and *MYB101* genes were strongly induced by *X. citri* infection compared with the non-pathogenic strains in *C. limon*. Nevertheless, the expression of *NCED3* and *csi-miR159* genes increased at 24 hpi, whereas *MYB101* expression levels remained repressed, in response to *Xcc* and *Xfa* (Fig. 6b). These results suggest that, in *C. limon* NHR and HDR, the ABA biosynthesis and response pathways are repressed in the early period of the defence response, when compared with the compatible interaction with *X. citri*.

Elicited NHR and HDR are effective against *X. citri*

In order to test whether the NHR and HDR, triggered by *Xcc* and *Xfa*, respectively, are able to protect *C. limon* plants against pathogenic *X. citri* and control canker development, young leaves were pre-inoculated by cotton swab with bacterial suspensions of *GFP*-tagged *Xcc* and *Xfa*, according to Roeschlin *et al.* (2017). Two different protection assays were performed, at 2 and 7 days post-treatment (dpt), to examine whether the putative protection is maintained. At 2 and 7 dpt, the pre-inoculated leaves were challenged with *X. citri-GFP* by spraying (Fig. 7a). Eighteen days after inoculation with *X. citri*, a reduction in

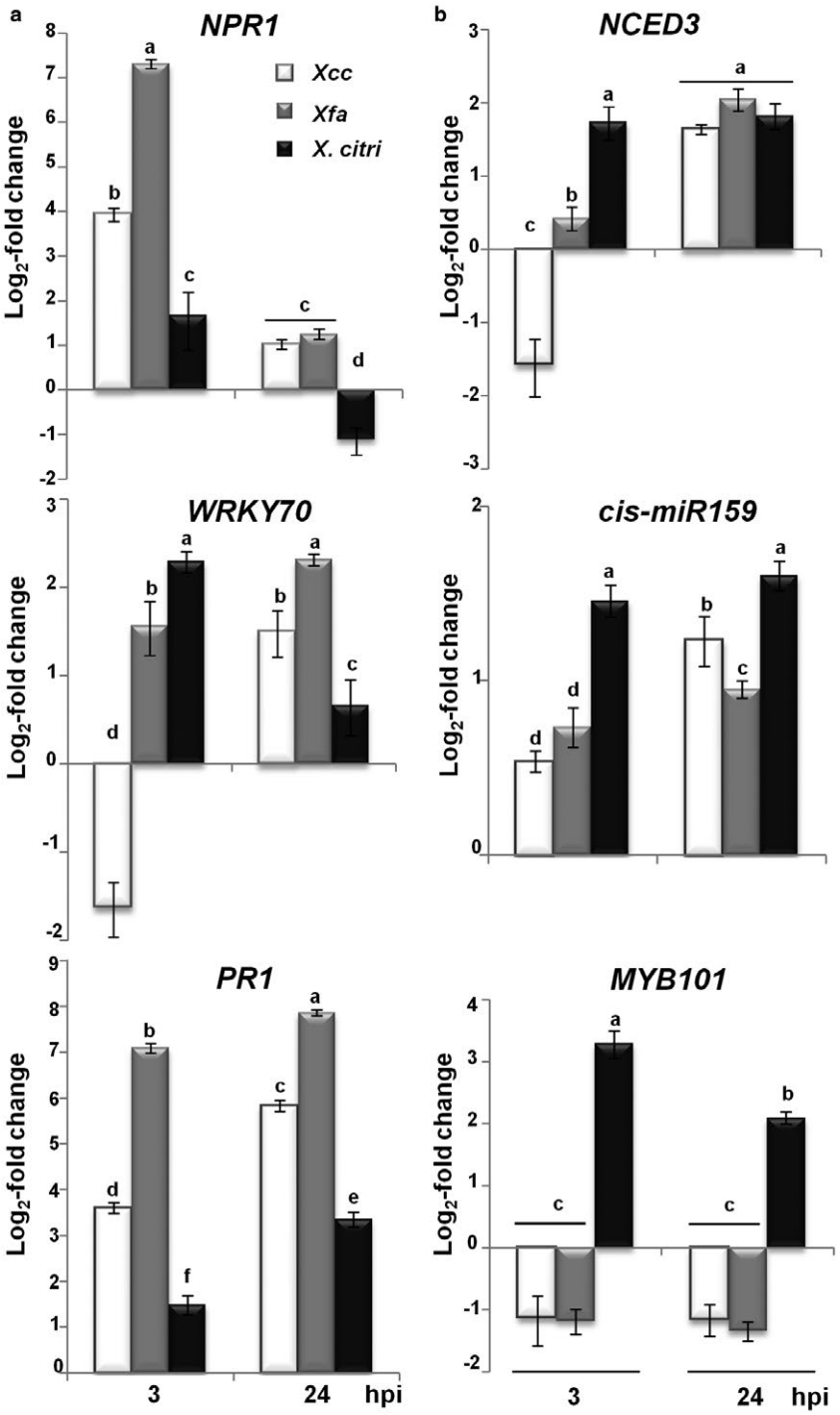

Fig. 6 Salicylic acid (SA) signalling pathway is induced, whereas abscisic acid (ABA) synthesis and signalling are repressed, in *Citrus limon* non-host resistance (NHR) and the host defence response (HDR). Quantitative reverse transcription-polymerase chain reaction in leaves inoculated by pressure infiltration with bacterial suspensions [10^7 colony-forming units (CFU)/mL] of *Xanthomonas campestris* pv. *campestris* (*Xcc*), *X. fuscans* ssp. *aurantifolii* (*Xfa*) strain C and *X. citri* ssp. *citri* (*X. citri*). mRNAs were measured at 3 and 24 h post-inoculation (hpi). Relative gene expression ($\Delta\Delta$Ct) fold change of mRNA levels was performed considering mock-treated plants as reference sample and histone *H4* transcript as an endogenous control. Values are expressed as means ± standard deviation (SD) from three independent biological replicates. Different letters indicate significant differences at $P < 0.05$ [two-way analysis of variance (ANOVA), Tukey's test]. (a) Expression profiles of SA signalling pathway genes encoding non-expressor of pathogenesis-related genes 1 (*NPR1*), *WRKY70* transcription factor and pathogenesis-related 1 (*PR1*). (b) Expression profiles of ABA biosynthesis (*9-cis-epoxycarotenoid dioxygenase*, *NCED3*) and signalling (*MYB101* and *csi-miR159*) encoding genes.

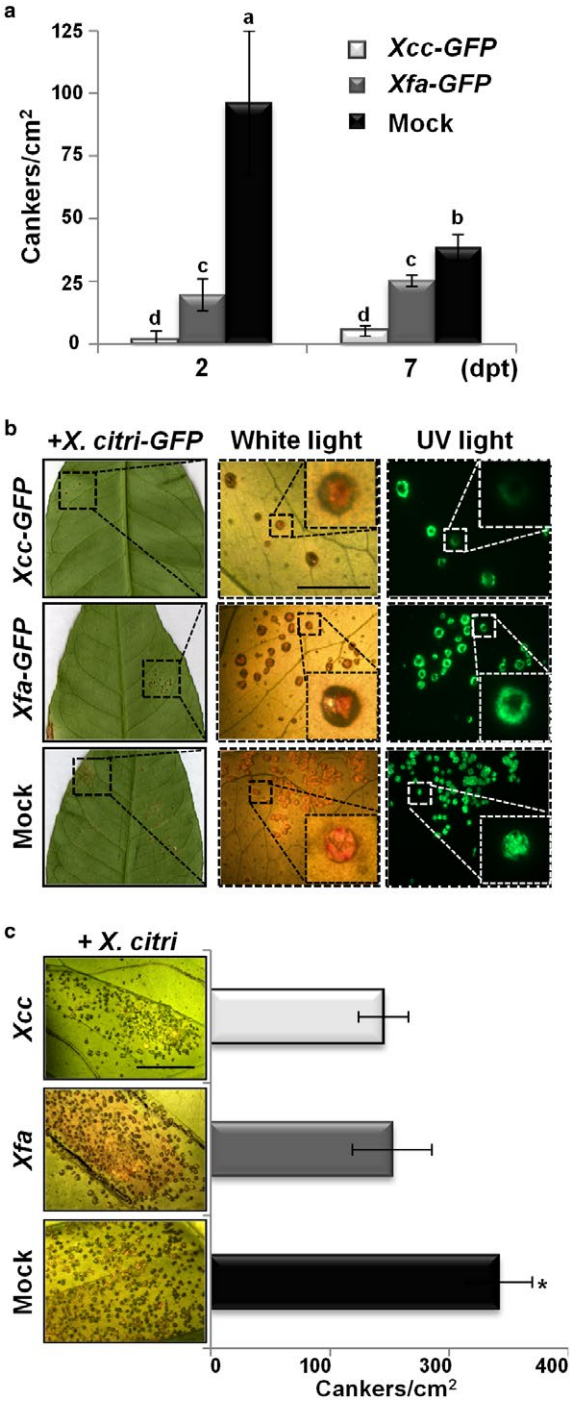

Fig. 7 Non-host resistance (NHR) and the host defence response (HDR) protect *Citrus limon* from canker development. (a) Number of canker lesions per square centimetre in leaves pretreated by cotton swab with bacterial suspensions [10^9 colony-forming units (CFU)/mL] of *Xanthomonas campestris* pv. *campestris* (*Xcc*) and *X. fuscans* ssp. *aurantifolii* (*Xfa*) strain C tagged with green fluorescent protein (*GFP*), or 10 mM $MgCl_2$ (mock). At 2 and 7 days post-treatment (dpt), the leaves were challenged via spraying with the pathogenic *X. citri* ssp. *citri* (*X. citri*)-*GFP* (10^9 CFU/mL), and canker lesions were quantified at 18 days post-inoculation (dpi). Values are expressed as means ± standard deviation (SD) from three independent biological replicates. Different letters indicate significant differences at $P < 0.05$ [two-way analysis of variance (ANOVA), Tukey's test]. (b) Phenotypic response of *C. limon* leaves pretreated with *Xcc-GFP*, *Xfa-GFP* or mock, and subsequently challenged with *X. citri-GFP* strain at 2 dpt, as described in (a). Sections from the left panels are shown magnified in the right panels under white and UV light. (c) Phenotypic response and canker quantification (18 dpi) of *C. limon* leaves pretreated by pressure infiltration with *Xcc-GFP* and *Xfa-GFP* bacterial suspensions (10^6 CFU/mL) or mock, and subsequently challenged (2 dpt) via infiltration with *X. citri-GFP* (10^6 CFU/mL). Scale bar, 10 mm. [Colour figure can be viewed at wileyonlinelibrary.com]

canker development was observed in leaves pre-inoculated with both non-pathogenic bacteria in both assays. Interestingly, the NHR induced a greater protection than the HDR, indicated by the small number of cankers/cm^2 developed by *X. citri* (Fig. 7a). In addition, the phenotype of canker lesions developed by *X. citri* was different between control (mock-inoculated) leaves and leaves pretreated with either *Xcc* or *Xfa*. In previously inoculated tissue, the cankers were of the vesicle type, whereas, in leaves with mock treatment, the cankers were of the corky type. In particular, in leaves pre-inoculated with *Xcc*, most of the lesions showed arrested growth of *X. citri* (diminished GFP fluorescence inside the lesions) (Fig. 7b). These results indicate that the NHR and HDR protect *C. limon* from canker development over an extended period. Remarkably, the NHR was more effective than the HDR in suppressing the action of pathogenicity effectors, such as PthA4, from *X. citri*. Moreover, when bacterial inoculations, pre-inoculations and pathogenic *X. citri* inoculations were performed by pressure infiltration, bypassing its entry through stomata, *Xcc* triggered a similar level of protection to *Xfa* against canker development (Fig. 7c). These results indicate that the leaf preformed defences were not critical in this NHR, and confirm that stomata-mediated plant defence plays a key role in the protection triggered by *Xcc*.

Citrus limon NHR triggered by *Xcc* is independent of type III effector proteins

The type III effector AvrGf2, secreted by *Xfa*, is responsible for triggering HDR in *Citrus* spp., except in *C. aurantifolia*. AvrGf2 belongs to the XopAG effector family which is present in several xanthomonads, including *Xcc* (Gochez *et al.*, 2017). To further determine whether the NHR triggered by *Xcc* involves type III effector proteins, the *hrcV*-deficient mutant strain of *Xcc* 8004 (*Xcc* Δ*hrcV*), defective in the T3S system (Cerutti *et al.*, 2017), was assayed. Regardless of the method used for bacterial inoculation in *C. limon*, *Xcc* Δ*hrcV* developed an asymptomatic phenotype and bacterial population growth similar to wild-type *Xcc* (data not shown). In addition, the microscopic cell death response was

comparable with that observed in NHR triggered by wild-type *Xcc* (Fig. 8a). Furthermore, the protection assays, performed as described previously, indicated that the *Xcc* Δ*hrcV* strain protects in a similar manner to wild-type *Xcc*, suggesting that T3S effectors of *Xcc* are not involved in this NHR (Fig. 8b).

DISCUSSION

In this work, we investigated the mechanisms of NHR in *C. limon* triggered by the brassica pathogen *Xcc*, a response of the plant immune system that renders the plant resistant to the canker-causing *X. citri*, and compared this response to the HDR triggered by *Xfa*. We demonstrated that *Xcc* induces type I NHR, which appears to be independent of *C. limon* preformed defence barriers, as the asymptomatic phenotype and bacterial population growth are similar when the bacterium is sprayed or infiltrated into the apoplast. The presence of plant-associated biofilms is correlated with *Xanthomonas* spp. pathogenicity (Bianco *et al.*, 2016; Roeschlin *et al.*, 2017; Vojnov and Marano, 2015). The induction of NHR and HDR triggered by *Xcc* and *Xfa*, respectively, interferes with bacterial biofilm development, which is associated with the arrest of bacterial growth on *C. limon* leaves. The disruption of the biofilm was also shown in the HDR triggered by a natural variant of *X. citri* (Roeschlin *et al.*, 2017).

Interestingly, *Xcc* elicits a sustained stomatal closure that is more effective than that induced by *Xfa* at 4 hpi, thus avoiding massive pathogen invasion of the apoplast. Notwithstanding the basal level of bacterial entry, as there is no macroscopic HR, *Xcc* appears to survive, but not multiply, in citrus tissue. In *C. sinensis* and *C. reticulata*, *Xcc* was isolated from the endophytic bacterial communities (Araujo *et al.*, 2002). In rice, it was observed that bacterial endophytes decrease stomatal conductance and stomatal density under water deficit (Rho *et al.*, 2018). Furthermore, non-adapted pathogens also induce a sustained stomatal closure during infection of Arabidopsis and tomato (Lee *et al.*, 2013; Melotto *et al.*, 2006). However, in Arabidopsis, *Xcc* is able to manipulate stomatal movements to gain access into the host leaf by the production of a secreted factor that is regulated by quorum sensing (Gudesblat *et al.*, 2009). This molecule remains to be identified, and therefore it is not known whether it is produced during the NHR interaction with *C. limon*, or whether it is even functional in this different plant. It should be noted that, in agreement with our results in *C. limon*, *X. citri* can antagonize ABA-dependent stomatal closure in *C. sinensis*. This effect is mediated through the production of a mimic of a plant natriuretic peptide (Gottig *et al.*, 2008).

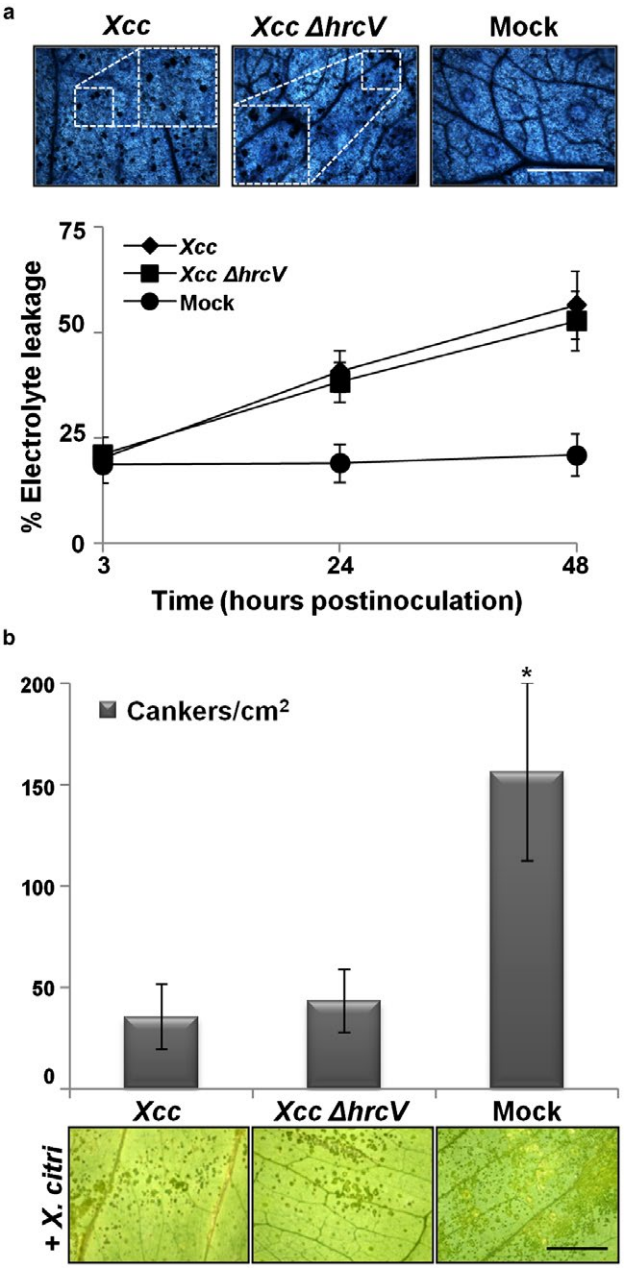

Fig. 8 *Citrus limon* non-host resistance (NHR) to *Xanthomonas campestris* pv. *campestris* (*Xcc*) is independent of type III effector proteins. (a) Microscopic cell death phenotype observed at 48 h post-inoculation (hpi) in leaves inoculated by cotton swab with bacterial suspensions [10^9 colony-forming units (CFU)/mL] of *Xcc* or the *hrcV*-deficient mutant strain of *Xcc* 8004 (*Xcc* Δ*hrcV*), or 10 mM $MgCl_2$ (mock). Insets show the amplification of microscopic cell death. Scale bar, 150 mm. Quantification of cell death in leaves treated as described previously by measurement of percentage electrolyte leakage at 3, 24 and 48 hpi. Values are expressed as means ± standard deviation (SD) of three independent biological replicates. (b) Number of canker lesions per square centimetre in leaves pretreated by cotton swab with *Xcc* and *Xcc* Δ*hrcV* bacterial suspensions (10^9 CFU/mL) or mock. At 2 days post-treatment (dpt), the leaves were challenged via spraying with the pathogenic *X. citri* ssp. *citri* (*X. citri*) (10^9 CFU/mL) and canker lesions were quantified at 18 days post-inoculation. Values are expressed as means ± SD from three independent biological replicates. The dataset marked with an asterisk is significantly different as assessed by Tukey's test ($P < 0.05$). Scale bar, 10 mm. [Colour figure can be viewed at wileyonlinelibrary.com]

We also showed that NHR and HDR involve a large accumulation of H_2O_2 in *C. limon.* In particular, the early *SOD2* gene expression induced by *Xcc* was associated with a higher accumulation of H_2O_2, initially in the guard cells promoting stomatal closure, and the absence of $O_2^{\bullet-}$. In Arabidopsis, PAMP-mediated H_2O_2 production requires the NADPH oxidase RBOHD (respiratory burst oxidase homologue protein D), superoxide dismutases and apoplastic peroxidases (Arnaud and Hwang, 2015). It is well known that ROS is a key signal messenger in the plant defence responses leading to stomatal closure, cell wall reinforcement (including callose deposition), accumulation of phenolic compounds and induction of defence gene expression within and surrounding the infected cells (O'Brien *et al.*, 2012; Qi *et al.*, 2017; Singh *et al.*, 2017). Interestingly, in Arabidopsis, *X. citri* triggered the accumulation of H_2O_2, which was associated with type I NHR in this non-host (An and Mou, 2012). Moreover, in *N. benthamiana*, the type II NHR triggered by *X. oryzae* pv. *oryzae* also accumulates H_2O_2, but not $O_2^{\bullet-}$, prior to the appearance of the HR (Li W *et al.*, 2012).

On the other hand, the induction of *PAL1*, *CYP83B1* and *cis-miR393* in *C. limon* against *Xcc* and *Xfa* suggests that cell wall reinforcement is a fundamental mechanism in both defence responses, limiting pathogen invasion, although at different timing and intensity. In addition, the accumulation of phenolic compounds and the induction of the SA signalling pathway are lower in NHR than in HDR. Consequently, the higher levels of *PAL1*, the main enzyme of phenylpropanoid metabolism which plays a key role in lignin and SA biosynthesis and the production of phenolic compounds, elicited by *Xcc*, may be associated with a redirection to lignin metabolism and thickening of the cell wall. In *Citrus* spp., the induction of *PAL1* has also been reported during different HDR (Cernadas *et al.*, 2008; Roeschlin *et al.*, 2017), and, in Arabidopsis, a fast and increased expression of the lignin biosynthesis genes contributes to NHR against *Pseudomonas syringae* (Mishina and Zeier, 2007). This defence strategy could also be linked to the higher accumulation of H_2O_2 observed in this NHR, as suggested by Zhang *et al.* (2011).

Likewise, the increased levels of *CYP83B1* and *cis-miR393* induced by *Xcc* may be associated with the maintenance of stomatal closure and also cell wall reinforcement, through callose deposition observed in this interaction, controlling the entry and proliferation of the bacteria into *C. limon* tissue. In addition, flg22 perception induces the production of *miR393*, which represses auxin signalling (Navarro *et al.*, 2006). Therefore, the increased expression levels of *cis-miR393* in *C. limon* NHR to *Xcc* may also be associated with this repression of hormone signalling. In support of this hypothesis, in *C. sinensis*, the expression of genes involved in auxin biosynthesis and signalling pathways, including the auxin signalling F-box receptor (*AFB2*) gene, was down-regulated during the NHR to *Xcv* (Petrocelli *et al.*, 2018). Moreover, the overexpression of *miR393* increases resistance to *Pseudomonas syringae* pv. *tomato* DC3000 (PstDC3000) through the redirection of secondary metabolite biosynthesis towards the glucosinolate pathway (Robert-Seilaniantz *et al.*, 2011). In Arabidopsis, *CYP83B1* is mainly implicated in IGS biosynthesis and is induced in response to PstDC3000. Moreover, the synthesis of IGS is involved in stomatal closure and is required for callose deposition in response to flg22 (Bednarek, 2012; Clay *et al.*, 2009).

Accumulating evidence has shown that the defence hormone SA not only promotes ETI, but also contributes to NHR (Lee *et al.*, 2017). Here, *Xcc* recognition induces in *C. limon* the early expression of *NPR1*, *WRKY70* and *PR1*, suggesting that the SA signalling pathway is involved in this NHR. Nevertheless, the lower levels observed in SA signalling pathway-related genes in the NHR may be associated with the microscopic cell death response and asymptomatic phenotype triggered by *Xcc* in *C. limon*. Meanwhile, *Xfa*, which induces higher levels of SA signalling pathway genes, triggers macroscopic HR. It has been proposed that the induction of the SA signalling pathway in HDR to a non-pathogenic variant of *X. citri* is associated with the beginning of programmed cell death in *C. limon* (Roeschlin *et al.*, 2017). In addition, in *C. sinensis* NHR to *Xcv*, genes involved in SA signalling, such as *PR1*, were up-regulated (Daurelio *et al.*, 2013). In Arabidopsis, SA signalling mutants showed compromised resistance to non-adapted *X. citri* (An and Mou, 2012). In addition, functional SA signalling, involving the transcription factor NPR1, is required for stomatal closure induced by flg22 or ABA (Toum *et al.*, 2016; Zeng and He, 2010), supporting our hypothesis that stomatal immunity is crucial in *C. limon* NHR to *Xcc*.

In this work, ABA biosynthesis (*NCED3*)- and signalling (*MYB101*)-related genes are repressed in the *C. limon* NHR against *Xcc*. Conversely, these genes are strongly induced by pathogenic *X. citri* from the start of the infection, suggesting that ABA favours *C. limon* susceptibility to *X. citri*, contributing to canker development. In addition, according to our results, *csi-miR159*, acting as a negative regulator of the ABA response in Arabidopsis (Curaba *et al.*, 2014; Dubos *et al.*, 2010; Reyes and Chua, 2007), would be implicated in the regulation of the ABA response by decreasing *MYB101* levels in *C. limon* NHR and HDR. Meanwhile, in the pathogenic interaction, the *MYB101* transcription factor would not be regulated by *csi-miR159*. Supporting this, in Arabidopsis, it has been suggested that pathogenic bacteria deliver effectors by the T3S system into host cells to suppress miRNA regulatory pathways (Padmanabhan *et al.*, 2009). Interestingly, several ABA-related genes, including *NCED3*, were found to be down-regulated in *C. sinensis* NHR to *Xcv*, and this was also associated with a strong decrease in ABA levels at early times post-inoculation (Petrocelli *et al.*, 2018). In Arabidopsis and rice, several reports have shown that ABA accumulation induced by bacterial pathogens promotes susceptibility via the suppression of the accumulation of phenolic compounds through

phenylalanine ammonia-lyase (PAL) inhibition, callose deposition and also SA-mediated defences, contributing to symptom development (Gupta *et al.*, 2017; Lievens *et al.*, 2017; de Torres Zabala *et al.*, 2009; Xu *et al.*, 2013). However, ABA can also positively influence disease resistance by regulating stomatal closure (Lim *et al.*, 2015).

Our results show that *C. limon* NHR to *Xcc* shares several defence signalling components with the HDR to *Xfa*, notwithstanding that the differences highlighted throughout this work could be responsible for the greater protection elicited by *Xcc* against *X. citri* when bacterial pretreatments were performed by cotton swab. These results confirm that the preformed defences are not critical in these responses, and also that immunity-mediated stomatal closure plays an important role in the NHR to *Xcc*. Furthermore, this response is independent of T3S effectors of *Xcc*, suggesting that this NHR is based on PTI. Supporting this hypothesis, the long phylogenetic distance between Brassicaceae, the natural hosts of *Xcc*, and *Citrus* spp. increases the relative effectiveness of PTI compared with the contribution of ETI, as postulated by Schulze-Lefert and Panstruga (2011).

Based on these results, we propose that *Xcc*, acting as an endophyte inside the plant, has the potential to maintain activated the inducible defence responses. It has been observed that endophytic bacteria act by inducing priming and protecting plants against biotic and abiotic stress (Kandel *et al.*, 2017; Pavlo *et al.*, 2011). It has been reported that bacterial endophytes increase defence enzymes, such as PAL, and antioxidant activity, such as superoxide dismutase (SOD), and enhance callose deposition and SA-responsive genes in host-treated plants (Liu *et al.*, 2017; Mishra *et al.*, 2018).

This is the first report of the characterization of a type I NHR in a non-model citrus plant at the physiological, biochemical and molecular level, which was simultaneously compared with the host and canker disease responses. Our results indicate the possibility to exploit and take advantage of the *C. limon* immune system for the sustainable management of citrus canker disease.

CONCLUSION

The *Brassica* pathogen *Xcc* triggers asymptomatic non-host resistance in *Citrus* spp., associated with a range of defence-related responses, and protects the plant from citrus canker development.

EXPERIMENTAL PROCEDURES

Bacterial strains and growth conditions, and plant material and pathogenicity assays

Xcc strain 8004 (Daniels *et al.*, 1984), *Xfa* strain C-1473 (Chiesa *et al.*, 2013) and *X. citri* strain T (Roeschlin *et al.*, 2017) were grown as described by Siciliano *et al.* (2006), and were transformed by electroporation with plasmid pMP2444 expressing *GFP* (Rigano *et al.*, 2007). The *Xcc* strain 8004 mutant in the *hrcV* gene (*Xcc* Δ*hrcV*), defective in the T3S system, has been described previously (Cerutti *et al.*, 2017).

Lemon plants [*C. limon* (L.) Burm. f.], cultivars 'Eureka' and 'Genova', grafted onto Troyer citrange [*Poncirus trifoliata* (L.) Raf. × *C. sinensis* (L.) Osb.], were grown and conditioned for bacterial inoculation as reported previously (Favaro *et al.*, 2014). Bacterial suspensions of *Xanthomonas* strains in 10 mM $MgCl_2$ were inoculated by spraying, cotton swab or pressure infiltration onto 15-day-old leaves of new shoots (Favaro *et al.*, 2014; Roeschlin *et al.*, 2017). A 10 mM $MgCl_2$ solution was used as mock inoculation. Inoculated plants were kept in a growth cabinet. Symptom development and disease progression were phenotypically monitored and registered using an MVX10 stereomicroscope and photographed under white and UV light (520 nm), and through bacterial population growth (Chiesa *et al.*, 2013). Canker lesions were quantified per square centimetre using Image J software (v1.41; National Institutes of Health, Bethesda, MD, USA).

Figures 1–5, 7 and 8 show representative results from three independent experiments, each involving three different leaves from three different plants.

Biofilm analysis

Bacterial adhesion and biofilm formation on polystyrene microplates and bacterial adhesion on abaxial surfaces of 'Eureka' leaves were analysed by crystal violet staining, as described previously (Rigano *et al.*, 2007). Stained bacteria attached to the leaf surface were examined and photographed under white light using a microscope (BX50F4; Olympus Optical Ltd. Company, Tokyo, Japan). Biofilm formation *in planta* was examined using *GFP*-tagged *Xanthomonas* spp. and monitored through an inverted confocal laser scanning microscope (Favaro *et al.*, 2014).

Stomatal movement analysis

Well-watered 'Eureka' plants were exposed to light for at least 3 h at 150–200 μE/s/m^2, 70% humidity and temperatures ranging from 25 to 28 °C in a growth cabinet. Fully expanded young leaves were inoculated by spraying with bacterial suspensions. As controls, 20 mm ABA (mixed isomers; Sigma-Aldrich, St. Louis, MO, USA) and water were used. A dried-gel imprint or mark from the leaf epidermis was used to visualize and analyse stomatal movements (Horiguchi *et al.*, 2006). Briefly, a drop of contact adhesive was placed on a glass slide, and the abaxial surface of a leaf sample was immediately gently placed on it. Once the contact adhesive had solidified, the leaf material was carefully peeled off, and the remaining contact adhesive imprint was left to dry for about 30 min. The imprinted leaf epidermis was observed under a light microscope (BH2; Olympus Optical Ltd.

Company). For the different treatments and time points evaluated, photographs were taken of at least 10 random zones. The sizes of 60 random stomatal apertures were measured for each treatment, and three samples from each leaf and three leaves of two plants were collected in each replicate. The width of the stomatal aperture was measured using the software Image-Pro version 4.5 for Windows (Cybernetics Inc., Rockville, MD, USA).

Cell death, ROS, callose and phenolic compounds detection

Cell death was visualized in *C. limon* leaves after staining with lactophenol–trypan blue (Koch and Slusarenko, 1990; Roeschlin *et al.*, 2017). Cell damage was quantified as described by Sanchez *et al.* (2010), modified as follows. Four leaf discs (9 mm in diameter) were sampled from cotton swab-inoculated areas and floated in 15-mL tubes containing 5 mL of distilled water for 4 h at 25 °C with shaking. The conductivity was measured using an ion conductivity meter (Twin cond B-173; Horiba, Tokyo, Japan), referenced as value A. At the end of the assay, samples were boiled in distilled water for 15 min, and the conductivity was subsequently measured at room temperature (value B) to determine the percentage of electrolyte leakage as (value A/value B) × 100. The histochemical detection of $O_2^{\bullet-}$ and H_2O_2 in *C. limon* inoculated tissue was carried out by staining with nitroblue tetrazolium (NBT) (Sigma-Aldrich) (Zhang *et al.*, 2011) and the fluorescence marker 2′,7′-dichlorofluorescein diacetate (DCFH-DA; Sigma-Aldrich) (Chiesa *et al.*, 2013), respectively. Callose deposition was visualized by aniline blue staining (Enrique *et al.*, 2011), and callose deposits were quantified using the program Image J (v1.41; National Institutes of Health) and expressed as the average number of callose deposits per field of view (0.50 mm^2). Autofluorescence of phenolic compounds was observed by fluorescence microscopy (excitation at 450–490 nm; emission at 520 nm) (Chen *et al.*, 2012) using free-hand leaf sections (Lux *et al.*, 2005). Leaves were examined and photographed under UV light with an epifluorescence microscope (BH2; Olympus Optical Ltd. Company).

RNA preparation and quantitative reverse transcription-polymerase chain reaction (qRT-PCR) assays

Leaf discs from three independent inoculated *C. limon* plants were randomly harvested at different time points and considered as an independent biological replicate. Three independent biological replicates were performed. Total RNA was extracted using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA), following treatment with RQ1 RNase-free DNase (Promega, Mannheim, Germany). cDNA was synthesized from 2 μg of DNase-treated total RNA using Superscript III reverse transcriptase (Invitrogen) according to the manufacturer's instructions. For the miRNAs, the artificially designed stem-loop oligomers (SLOs) were used (Varkonyi-Gasic *et al.*, 2007). Oligo dT12-18 primers were used for mRNAs. cDNA from the reactions was diluted 1 : 20 with distilled water before qRT-PCR. The reaction was carried out in a 20-μL volume containing 5 μL of diluted cDNA, miRNA specific forward (FW) and universal reverse (RV) primers for miRNAs, or FW and RV primers for mRNAs, using Real Mix (Biodynamics SRL, BA, Argentina), and monitored in a Mastercycler® ep realplex system (Eppendorf, Hamburg, Germany). Primer sequences used for qRT-PCR are described in Table S1 (see Supp-orting Information). The miRNA amplification conditions were as follows: 95 °C for 2 min, and 40 cycles of 95 °C for 15 s, 52 °C for 30 s and 72 °C for 45 s. After amplification, a thermal denaturing cycle of 95 °C for 15 s, 60 °C for 15 s and 95 °C for 15 s was applied to determine the dissociation curves. The qRT-PCRs for mRNAs were performed according to Roeschlin *et al.* (2017). Technical triplicates for each sample were performed. Relative transcript abundance between samples was normalized against histone *H4* (Shiotani *et al.*, 2007) as an internal standard using the ΔΔCt method (Livak and Schmittgen, 2001). $MgCl_2$-treated (mock) *C. limon* leaves served as the reference sample.

Statistical analyses

Data were subjected to a two-way analysis of variance (ANOVA) and the treatment means were analysed using Tukey's test ($P < 0.05$) through SAS University Edition (2017) (SAS Institute Inc., Cary, NC, USA).

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AUTHOR CONTRIBUTIONS

M.A.C. and M.R.M. designed the research. M.A.C., R.A.R., M.A.F., F.U., L.C-B. and R.D'A. performed the research. M.A.C., J.G. and M.R.M. wrote the manuscript.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article at the publisher's web site:

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