Genetics and Genomics of Resistance

Different Responses in Mandarin Cultivars Uncover a Role of Cuticular Waxes in the Resistance to Citrus Canker

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ABSTRACT

'Okitsu' is a mandarin cultivar showing substantial resistance to *X. citri* subsp. *citri* (*X. citri*). We have previously shown that this cultivar has significantly lower canker incidence and severity than 'Clemenules', particularly during early stages of leaf development in the field. This differential response is only seen when the leaves are inoculated by spraying, suggesting that leaf surface contributes to resistance. In this work, we have studied structural and chemical properties of leaf surface barriers of both cultivars. Ultrastructural analysis showed a thicker cuticle covering epidermal surface and guard cells in young 'Okitsu' leaves than in 'Clemenules'. This thicker cuticle was associated with a smaller stomatal aperture and reduced cuticle permeability. These findings correlated with an accumulation of cuticular wax components, including primary alcohols, alkanes, and fatty acids. None of these differences were observed in mature leaves, where both cultivars are equally resistant to the bacterium. Remarkably, mechanical alteration of cuticular thickness of young 'Okitsu' leaves allows canker development. Furthermore, cuticular waxes extracted from young 'Okitsu' leaves have higher antibacterial activity against *X. citri* than 'Clemenules'. Taken together, these data suggest that a faster development of epicuticular waxes in 'Okitsu' leaves play a central role in its resistance to *X. citri*.

Keywords: bacteriology, canker disease resistance, cuticle, cuticular thickness, genetics and resistance, 'Okitsu' mandarin, stomatal defense, waxes, *Xanthomonas*

Plants have evolved multiple mechanisms to defend against pathogen invasion. The first line of defense is the plant surface composed by preformed structural barriers such as the cuticle, considered a specialized lipidic modification of the epidermal cell wall (Domínguez et al. 2011; Samuels et al. 2008; Yeats and Rose 2013). The cuticle covers the aerial parts of plants, which is dominated by the leaves forming the phyllosphere, and it serves as a key interface between plant and environment, protecting against invading pathogens and abiotic stresses (Aragón et al. 2017; Yeats and Rose 2013; Ziv et al. 2018). The plant cuticle is mainly composed of cutin, a lipid-derived polyester, and waxes, which are either embedded in the cutin matrix (intracuticular waxes) or deposited on its outer surface (epicuticular waxes) (Aragón et al.

2017; Domínguez et al. 2011; Samuels et al. 2008). The architecture and composition of the cuticle varies between plant species, organs and developmental stages. The molecular basis of cuticle biosynthesis, export and regulation has been extensively studied in the model plant *Arabidopsis*, as well as in crop plants, including tomato, maize, rice, citrus, and *Brassica napus* (Lee and Suh 2015; Liu et al. 2015; Samuels et al. 2008; Wang et al. 2014, 2016).

The cuticle is the first contact with bacteria when they land on the leaf surface. In order to cope with this defensive barrier, many foliar pathogenic bacteria have evolved the ability to adhere and develop biofilm on the host surface before gaining access into the intercellular spaces of the mesophyll tissue through stomata (Melotto et al. 2008; Rigano et al. 2007; Vojnov and Marano 2015). Plant genotype play a major role in determining the structure of the phyllosphere, interfering with the ability to develop biofilm, a key factor of bacterial pathogenicity (Bodenhausen et al. 2014; Favaro et al. 2014; Schlechter et al. 2019; Whipps et al. 2008). It has been demonstrated that thicker and less permeable cuticles might interfere with epiphytic bacterial colonization, inhibiting the wetting of the leaf surface and limiting solubilization and diffusion of nutrients from the leaf (Bodenhausen et al. 2014; Lindow and Brandl 2003; Schlechter et al. 2019). Moreover, stomatal density and structure could also play an important role as preformed physical barriers against bacterial infection (Gonçalves-Zuliani et al. 2016; Wang et al. 2011).

The second line of plant defense is triggered by the perception of conserved pathogen-associated molecular patterns by cell surface pattern-recognition receptors (PRRs) in the plasma membrane (Couto and Zipfel 2016). Activation of PRR-mediated response results in stomatal closure, limiting bacterial proliferation at early
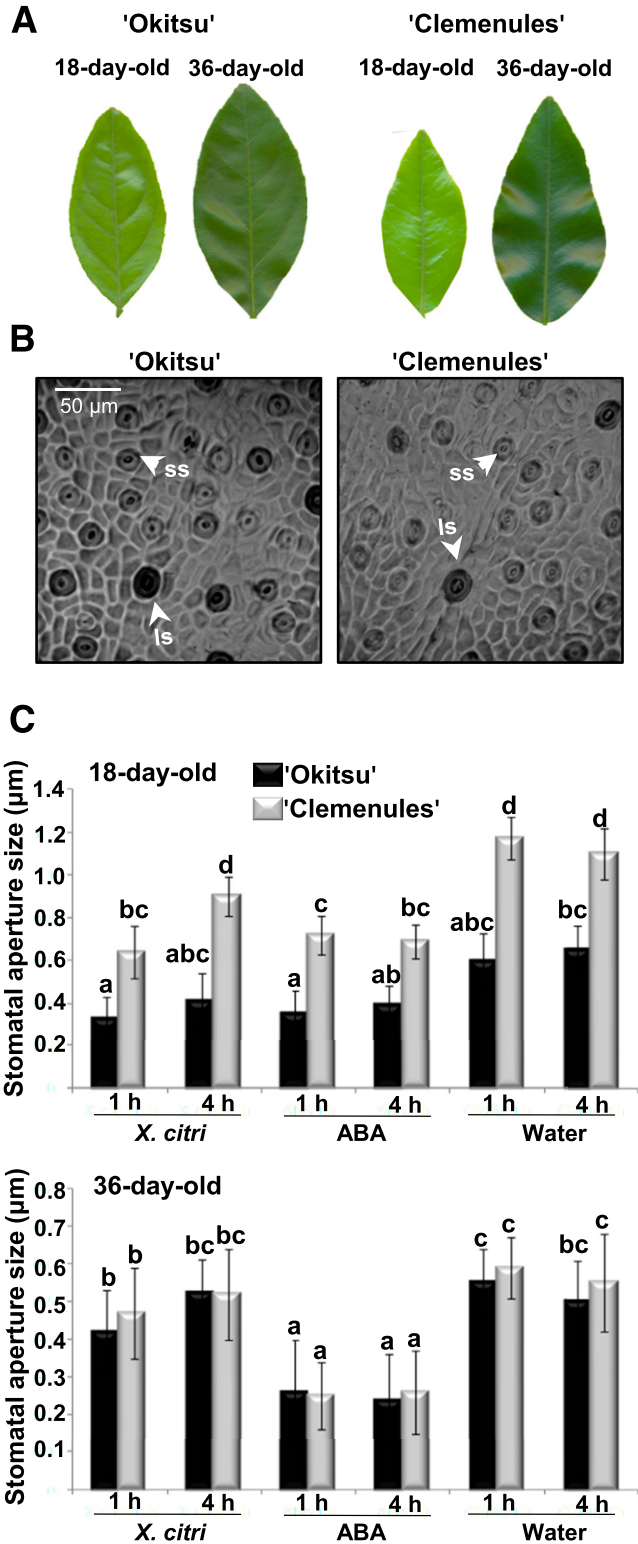
Fig. 1. Comparison of stomatal distribution and aperture size between 'Okitsu' and 'Clemenules' leaves. **A,** Phenological stages of young (18-day-old) and mature (36-day-old) 'Okitsu' and 'Clemenules' leaves. **B,** Dried-gel imprint of intact mandarin epidermis showing stomatal distribution in young leaves. ls, large stomata; and ss, small stomata. **C,** Quantification of stomatal aperture at 1 and 4 h postinoculation in leaves exposed to *Xanthomonas citri* infection and abscisic acid (ABA) or water treatments by spraying. Values are expressed as the means ± standard deviation from three independent experiments ($n = 50$ stomata). Different letters above the bars indicate significant differences at $P < 0.05$ (three-way analysis of variance, Tukey's test).

stages of infection. However, pathogenic bacteria can reverse the stomatal-based defense and fully colonize the host plant (Chiesa et al. 2019; Melotto et al. 2017). A further level of induced defense is initiated by host recognition of effectors that are secreted into cells, resulting in the triggering of multiple responses that lead to the arrest of bacterial growth (Cesari 2018; Toruño et al. 2016).

Xanthomonas citri subsp. *citri* (*X. citri*) is the causal agent of Asiatic citrus canker, a disease that seriously affects most of the world's commercial citrus cultivars. Infected fruits have decreased commercial quality, compromising the acceptance by most markets (Ference et al. 2018). Evaluations in field and controlled conditions suggest that several types of *Citrus* and closely related genera, including *C. ichangensis*, *C. junos*, *C. medica*, *C. mitis*; *C. unshiu*, 'Dalan Dalan' (a cultivar similar to *C. paradisi*), *Citrofortunella* spp., *Fortunella* spp., and 'Lakeland' limequat (*C. aurantifolia* × *F. japonica*) are resistant to *X. citri* (Chen et al. 2012; De Carvalho et al. 2015; Deng et al. 2010; Favaro et al. 2014; Gochez and Canteros 2008; Gonçalves-Zuliani et al. 2016: Graham et al. 1992; Lee et al. 2009; Shiotani et al. 2009; Viloria et al. 2004; Wang et al. 2011). However, in some of these pathosystems quantitative resistance present in field is fully or partially broken down when invasive inoculation methods, such as pressure infiltration or pin prick inoculation, are used. This suggests that preformed defenses at the plant surface might be involved in the resistance to bacterial invasion (Favaro et al. 2014; Gonçalves-Zuliani et al. 2016; Graham et al. 1992; Wang et al. 2011).

The mechanisms underlying plant preformed defense and its relevance for limiting bacterial pathogen entry to the apoplast remain poorly understood. It has been reported that smaller stomatal density, size and aperture, and also higher epicuticular wax content contribute to the differential response to *X. citri* infection between the resistant kumquat genotype 'Meiwa' and the susceptible navel orange genotype 'Newhall' (Wang et al. 2011). In addition, a smaller stomatal aperture was associated with a lower susceptibility to *X. citri* in 'Pera' IAC orange, but no differences were observed in the stomatal density between this genotype and the more susceptible 'Washington navel' (Gonçalves-Zuliani et al. 2016).

Previously, we have shown that the resistance to *X. citri* in *C. unshiu* 'Okitsu' was associated with a faster phenological development of the leaf during the period of maximal susceptibility to *X. citri* infection, which might be coincident with a rapid cuticle development (Favaro et al. 2014). In addition, the resistance to *X. citri* was evident in bacterial spray-inoculated plants but not in those inoculated by infiltration, suggesting that the leaf surface contributes to quantitative resistance, limiting bacterial epiphytic fitness and biofilm formation.

In this work, we have studied the structural and chemical properties of leaf surface barriers of two mandarin cultivars to shed light on the differences that lead to resistance or development of citrus canker disease. Our findings highlight the multiple functions of the thicker 'Okitsu' cuticle in limiting bacterial establishment, including small stomatal aperture, low water permeability and a fast development of cuticular waxes. Furthermore, the amount of cuticular waxes in the early stage of leaf development is associated with antimicrobial activity against *X. citri*.

MATERIALS AND METHODS

Plant material and bacterial inoculation. One-year-old 'Clemenules' (*C. clementina* Hort. ex Tan.) and 'Okitsu' (*C. unshiu* Marc.) plants, grafted onto *Poncirus trifoliata* (L.) Raf. rootstocks were kept in a growth chamber, with a temperature range of 25 to 28°C, high humidity (>95%), a 14 h photoperiod, and a light intensity of 150 to 200 m^{-2} $^{-1}$. New shoots of approximately 1 cm size with at least five leaves were selected after pruning the plants. All the leaves of the same shoot were considered to be of the same ontological age. Experiments described in this work were done in young (18-day-old) and mature (36-day-old) leaves (Fig. 1A).

These phenological development stages showed maximal and minimal differences between cultivars to canker disease (Favaro et al. 2014).

Bacterial suspensions of *X. citri* strain A^{E28} (10^7 CFU ml^{-1}) were prepared in 10 mM $MgCl_2$ and inoculated spraying mandarin leaves (Favaro et al. 2014). Inoculated plants were kept in a growth chamber.

Stomatal density and stomatal aperture analysis. Stomatal density, spatial distribution and aperture size were evaluated in abaxial epidermis imprints of the leaf, obtained using cyanoacrylate adhesive, according to Chiesa et al. (2019). Stomatal density was determined as the number of stomata per square millimeter. Spatial distribution of stomata was evaluated considering the predominant stomatal type and the relation between the number of large and small stomata.

To evaluate stomatal aperture, mandarin plants were exposed to light for at least 3 h at 150 to 200 m^{-2} $^{-1}$, 70% humidity, and temperatures ranging from 25 to 28°C before beginning the experiment. Leaves were inoculated with *X. citri* suspensions. As control of stomatal closure and opening, 20 µm ABA (mixed isomers; Sigma-Aldrich, St. Louis, MO, U.S.A.) and water were used, respectively.

Imprints of abaxial epidermis (sample) were observed under a light microscope (BH2; Olympus Optical Ltd. Company, Shinjuku, Tokyo, Japan). For the different treatments and times, photographs were taken of at least 10 random zones per sample. Eighteen samples were obtained from six leaves collected from two different plants. Three independent experiments were conducted yielding similar results. The width of 50 random stomatal apertures was measured for each treatment and time point, using the software Image J v 1.41 (National Institutes of Health, Bethesda, MD, U.S.A.).

Cuticular permeability. For assessing the permeability of mandarin leaf surfaces to water-soluble molecules, toluidine blue staining was adapted from a previously described protocol (Bessire et al. 2007) with some modifications. Tissue samples (five pieces of 1 cm^2 per leaf) were decolorized in 95% ethanol, equilibrated in 0.2 M $NaPO_4$ (pH 9.0) for 1 h, and incubated in 0.05% (wt/vol) toluidine blue solution for up to 6 h. Tissue samples treated with water and processed as described above were used as controls. Dye penetration to cells was examined with an Olympus BX50F4 microscope (Olympus Optical Ltd. Company). Toluidine blue is a polychromatic dye, therefore the color observed depends on the tissue staining (O'Brien et al. 1964). The samples were photographed in a Molecular Imager ChemiDoc TM XRS+ Imaging System (Bio-Rad, U.S.A.) and quantification of permeability was performed using a standard dye curve and the Quantity One software (Bio-Rad). Each experiment involved at least 15 samples per treatment, obtained from three different leaves, each one collected from different plants. Three independent experiments were conducted yielding similar results.

Transmission electron microscopy (TEM). The ultrastructure of the abaxial leaf cuticle was analyzed by TEM. Leaf sections (samples) from both mandarin cultivars were prepared and observed according to Roeschlin et al. (2017). Cuticle thickness was measured at three points of the epidermis using the Software Image J v 1.41 (National Institutes of Health). At least six different photographs were analyzed per leaf section. Samples from three different leaves, obtained from different plants were analyzed for each leaf age and genotype.

Total wax extraction. Cuticular wax from leaves surface was extracted as described by Beattie and Marcell (2002) with minor modification. A pool of young or mature leaves (150 cm^2, ~20 to 15 leaves according to phenological development) randomly selected from three different 'Okitsu' and 'Clemenules' plants were fully submerged in 50 ml of chloroform (Merck, Darmstadt, Germany). After stirring for 1 min at room temperature, the solvent was evaporated under a gentle stream of nitrogen. Total wax concentration

was expressed in micrograms per square centimeter of leaf area. The obtained samples were processed according to Wang et al. (2014) either for gas chromatography-mass spectrometry (GC-MS) analysis or antibacterial activity assays. Control (blank) samples were prepared in the same way as other samples, except that no leaves were added. Three independent experiments were conducted yielding similar results.

Wax analysis by GC-MS. Wax composition of cuticular waxes of mandarin leaves was analyzed according to Wang et al. (2014), using capillary GC (Agilent 7890B, Agilent Technologies, Santa Clara, CA, U.S.A.) coupled with MS detector (5977A, Agilent Technologies) and a HP-5ms UI capillary column (30 m, 0.25 mm inner diameter, 0.25 µm film thickness, Agilent Technologies). Helium was used as a carrier gas at a flow rate of 1 ml min^{-1}. The following parameters were employed: inlet temperature 250°C, MS transfer line temperature 280°C, ion source temperature 230°C, electron impact (EI) 70 eV, and *m/z* range 50 to 750. Wax components were identified using a mass spectral database (NIST MS Library SW Kit, 2011b, Agilent Technologies). The relative percentage of each compound was determined by dividing the integrated area of the peak of the specific ion for the compound in question (×100) by the summed value for the areas of all peaks (Chen et al. 2014). Three independent experiments were conducted yielding similar results.

RNA isolation and expression analysis of cuticle-associated genes. The transcriptional levels of seven genes related to wax biosynthetic pathways were analyzed by quantitative polymerase chain reaction (qPCR). Total RNA from mandarin leaves (100 mg) was isolated according to the manufacturer's instructions (NucleoSpin RNA, Macherey-Nagel, Dueren, Germany). Reverse transcription was performed by M-MLV reverse transcription (Invitrogen, Carlsbad, CA, U.S.A.) with 1 µg of DNase-treated total RNA and oligo-dT12-18 as primers. Synthesized cDNA was used for qPCR. Seven candidate genes potentially involved in mandarin cuticle formation were selected from the *C. clementina* genome database (https://phytozome.jgi.doe.gov/) based on the homology to corresponding Arabidopsis genes with known functions in leaf cuticular wax biosynthesis. The gene locus, function of gene product, primers sequences and PCR conditions are given in Table 1. The reactions were carried out with real-time PCR master mix (Biodynamics SRL, BA, Argentina), and monitored in a Mastercycler ep realplex system (Eppendorf, Hamburg, Germany). Relative transcript abundance between samples was normalized against *histone H4* (Shiotani et al. 2007) as an internal standard using the $\Delta\Delta Ct$ method (Livak and Schmittgen 2001). 'Clemenules' leaves served as the reference sample. Each assay was performed with three different samples, involving three different leaves from three different plants. Three independent experiments were conducted yielding similar results.

Mechanical removal of 'Okitsu' cuticular waxes and canker development. Epicuticular waxes were removed from the abaxial surface of 'Okitsu' 18-day-old leaves by cotton swab and with gum arabic, according to Marcell and Beattie (2002) and Gniwotta et al. (2005), respectively. For gum arabic treatment, an aqueous solution of the adhesive was applied onto the entire abaxial surface of the leaves using a small paintbrush. After 1 to 2 h, the gum arabic solution formed a dry and stable polymer film in which wax crystals were embedded and the film was extracted without damaging the leaves. Leaves subjected to both treatments and untreated 'Okitsu' and 'Clemenules' leaves were inoculated with *X. citri* suspensions (10^7 CFU ml^{-1}) by spraying onto the abaxial epidermis of mandarin leaves, according to Favaro et al. (2014). Inoculated plants were kept for 30 days in a growth chamber. All plant inoculations involved a minimum of two shoots with at least five leaves from each plant and four plants for each mandarin cultivar. Disease progression was monitored phenotypically and through analysis of bacterial growth curves. Fifteen days post-inoculation (dpi) samples were processed for TEM analysis

(Roeschlin et al. 2017). For determination of bacterial population, three leaf disks of 1 cm^2 were selected randomly from the inoculated leaves. The disks were immersed in 500 µl of 10 mM MgCl$_2$ in Eppendorf microfuge tubes. Bacterial cells were collected by homogenization of tissue with a plastic pestle. The suspension was stirred at room temperature for 5 min and serial dilutions of this suspension were plated on 1.8% agar NYG medium (peptone extract at 5 g liter^{-1}, yeast extract at 3 g liter^{-1}, and glycerol at 20 g liter^{-1}) supplemented with 100 g ml^{-1} ampicillin, to estimate the total bacterial numbers. Leaf samples were taken during 1 week to determine bacterial population sizes on the leaves. At each time point, samples were determined from three separate experiments and each experiment was measured in triplicate. Three independent experiments were conducted yielding similar results.

Antibacterial activity assays of cuticular waxes. Microbicidal properties of cuticular waxes from mandarin leaves were tested in vitro against *X. citri*. A pool of young leaves (150 cm^2, ~20 leaves) randomly selected from three different 'Okitsu' and 'Clemenules' plants were fully submerged in 50 ml of chloroform (Merck, Darmstadt, Germany) and processed as described above. The extracted cuticular waxes were dissolved in 200 µl of chloroform (Merck) and used in the screening of antibacterial activity on NYG agar plates. Spots of approximately 50 µl of each wax extract (corresponding to the wax present in 37.5 cm^2 of leaf) were deposited in triplicate onto 1.8% agar NYG supplemented with ampicillin at 100 g ml^{-1}. As control, spots of 50 µl of chloroform were deposited onto agar plates. Ten microliters of *X. citri* suspensions (10^4 CFU ml^{-1} in 10 mM MgCl$_2$) was inoculated over the dry wax extracts and control. The plates were incubated at 28°C for 72 h and the colonies grown on each spot were counted using the Software Image J v 1.41 (National Institutes of Health). Three independent experiments were conducted yielding similar results.

For determination of bactericidal activity in NYG liquid medium, the assay was adapted from the method described by Golus et al. (2016). Cuticular waxes from young leaves (150 cm^2) were extracted and processed as described above. The extracted cuticular waxes were dissolved in 15 µl of dimethylsulfoxide (DMSO, Merck), and 2 µl of this extract was mixed with 200 µl of *X. citri*

suspension (10^6 CFU ml^{-1} in 10 mM MgCl$_2$) and deposited in triplicate on 96-well Clear Flat Bottom Polystyrene TC-treated Microplates (Corning, U.S.A.). As control of bacterial growth, 2 µl of DMSO was mixed with 200 µl of *X. citri* suspension (10^6 CFU ml^{-1} in 10 mM MgCl$_2$). The plate was incubated at 28°C, and bacterial growth was assessed by microplate reader (Bio Tek Synergy 2 Multi-Detection, U.S.A.) at 600 nm. Three independent experiments were conducted yielding similar results.

Statistical analyses. Data were analyzed according to Student's *t* test ($P < 0.05$) through InfoStat Software v2017 (Di Rienzo et al. 2017), excepting stomatal aperture and bacterial population data that were subjected to a three-way analysis of variance (ANOVA: cultivar, time, and treatment) and one-way ANOVA, respectively. In both cases, the means were analyzed using Tukey's test ($P < 0.05$).

RESULTS

The stomatal pore aperture is smaller in young 'Okitsu' leaves than in 'Clemenules'. In order to investigate the role of stomatal density and size in the resistance to *X. citri*, imprints of abaxial epidermis of 18-day-old leaves of both mandarin cultivars were analyzed (Fig. 1A). No apparent qualitative differences were observed in the disposition of guard cells in relation to the subsidiary cells, showing both cultivars the same stomatal complex (anomocytic type, Fig. 1B). Moreover, the relation between the number of large and small stomata was similar to that reported for other *Citrus* species (Graham et al. 1992). In addition, no differences were observed in the stomatal density, indicated by the number of stomata per square millimeter between 'Okitsu' and 'Clemenules' (943 ± 64 and 1,004 ± 41, respectively, $P < 0.05$, Student's *t* test).

In order to investigate the role of stomatal response to *X. citri* in both mandarin cultivars during the different phenological leaf stages (young and mature leaves), bacterial promotion of stomatal closure was analyzed at 1 and 4 h postinoculation (hpi). ABA and water were used as control of stomatal closure and opening, respectively (Chiesa et al. 2019; Melotto et al. 2008). Interestingly, stomatal aperture was significantly smaller in 'Okitsu' than in

TABLE 1. Primers of mandarin cuticle-associated genes involved in wax biosynthesis used for qPCR analysis[a]

Gene	Gene product	Arabidopsis accession number[b]	*Citrus clementina* accession number[c]	Primer sequence (5′→3′)
CER1	VLC-aldehyde decarbonylase involved in alkane-forming pathway	At1g02205	Ciclev10019279m	TCACAGTTTCCACCAAATGA CGTAACCACATTCGTGTTCG
CER3[d]	VLC-acyl-CoA reductase involved in aldehyde and alkane-forming pathway	–	–	CAAGCAGCTCAACATTCCAA ATCGTCAGGCAATCTCATGG
CER4	Fatty acyl-CoA reductase involved in alcohol-forming pathway	At4g33790	Ciclev10028507m	GCTTCCTTGGAGACGTGAAG CGGTAGGCGTAATCCTGAAG
CER6	β-keto acyl-CoA synthase involved in fatty acid elongation	At1g68530	Ciclev10031329m	AGCTCGTAATCTTCTCCGCC TGCAGCCCATACCCGAAAG
CER7	3′-5′ exoribonuclease involved in the regulation of total wax loads	At3g60500	Ciclev10015159m	TAGGAGGCCTGAATGCTCAC GCTTCTTCGTGGTGAGTTGG
FAR2[e]	Fatty acyl-CoA reductase involved in alcohol-forming pathway	–	–	GAAAGTCAGTAGAGCAAGCGAAGC TTCCAGTCAATGCTCCCCAC
WIN1	AP2-EREBP-type transcriptional factor that activates cuticular wax biosynthesis by up-regulation of *CER1* and other genes	At1g15360	Ciclev10027305m	GTCATCACCAACGGAGAAGG TGAGGGATGGAGATGGAGAC
H4[f]	Histone H4	–	Ciclev10029640m	AGGCAAGGGATTGGGAAAGG AGAGCGTAAACGACGTCCATC

[a] *CER1*, eceriferum 1; *CER3*, eceriferum 3; *CER4*, acyl-CoA reductase; *CER6*, β-ketoacyl-CoA synthase KCS6/eceriferum 6; *CER7*, eceriferum 7; *FAR2*, acyl-CoA reductase; and *WIN1*, wax inducer 1 transcription factor. qPCRs were performed for 40 cycles according to the following conditions: denaturation at 95°C for 15 s, annealing at 57°C for 30 s, and extension at 72°C for 40 s. After amplification, melting-curve analyses were performed to exclude artifactual amplifications.
[b] Arabidopsis genome database (http://www.arabidopsis.org).
[c] *C. clementina* genome database (http://www.phytozome.net).
[d] Matas et al. (2010).
[e] Liu et al. (2015).
[f] Shiotani et al. (2007).

'Clemenules' at 1 hpi in 18-day-old leaves either for bacterial inoculation or control treatments (Fig. 1C). Moreover, at 4 hpi, *X. citri* reversed stomatal closure in 'Clemenules' whereas 'Okitsu' stomatas remained closed. Nevertheless, in mature leaves (36-day-old), stomatal aperture and response to bacterial infection or treatments were similar in both mandarin cultivars (Fig. 1C).

These findings suggest that a smaller stomatal aperture in young 'Okitsu' leaves may be one component of the surface barriers that contributes to the resistance to *X. citri* infection.

'Okitsu' show reduced cuticle permeability in young leaves. We previously demonstrate that young 'Okitsu' leaves interfere with plant-associated bacterial biofilms required for *X. citri* pathogenicity and canker development (Favaro et al. 2014). We hypothesized that the inhibition of bacterial adhesion and biofilm formation may be associated with a reduced cuticle permeability limiting solubilization and diffusion of nutrients from the leaf in this mandarin cultivar. To test this, permeability of the cuticle of 18-day-old leaves was assessed with toluidine blue and monitored over 6 h. After 30 min, only 'Clemenules' epidermal tissue showed accumulation of the dye (blue staining) within the guard cells and cell wall junctions (Fig. 2). Six hours after incubation with the staining solution, 'Clemenules' showed a deep penetration of the dye to subepidermal leaf tissue, indicated by the characteristic reddish staining of parenquimatic tissue. The percentage of cuticular permeability to the dye was 1.5-fold higher in 'Clemenules' than in 'Okitsu' ($58.0 \pm 4\%$ and $37.2 \pm 3\%$, respectively, $P < 0.05$, Student's *t* test), suggesting a negative relation between permeability and resistance to *X. citri*. As expected, the percentage of dye penetration was similar in 36-day-old leaves of both mandarin cultivars ($31.8 \pm 4\%$ and $35.6 \pm 2\%$, respectively).

A thick and deeper extending cuticle covers epidermal surface and guard cells in young 'Okitsu' leaves. To gain insight into the relationship between cuticular permeability and thickness in the resistance to *X. citri* the ultrastructure of the cuticle in both mandarin cultivars was analyzed by TEM during the main phenological stages of the leaf that show clear differences in susceptibility to *X. citri* infection (Fig. 3). Eighteen-day-old 'Okitsu' leaves showed a thicker cuticle than 'Clemenules', which covers the pavement epidermal cells and formed stomatal edges in the guard cells (Fig. 3A). In addition, in some areas 'Okitsu' cuticle penetrates deeply in the epidermal cell wall junctions, leading to the cuticularization of the anticlinal cell wall, generating a flat continuous layer on the epidermis. Conversely, in 'Clemenules', a thin cuticle covers the surface of epidermal cells, leaving depressions between epidermal cells junctions, designing a sinuous topography in the epidermal layer (Fig. 3A). In contrast, no differences in cuticle thickness were observed in mature (36-day-old) leaves between both mandarin cultivars (Fig. 3B).

Taken together, our data suggest that a faster cuticle development takes place in young 'Okitsu' leaves, where it may play a substantial role as a preformed physical barrier against *X. citri* infection.

The thick cuticle in young 'Okitsu' leaves is associated with an early accumulation of cuticular wax components. The plant cuticle waxes are predominantly composed of a mixture of aliphatic very long chain fatty acid (VLCFA) and their derivatives, as well as cyclic compounds including triterpenoid and sterols (Samuels
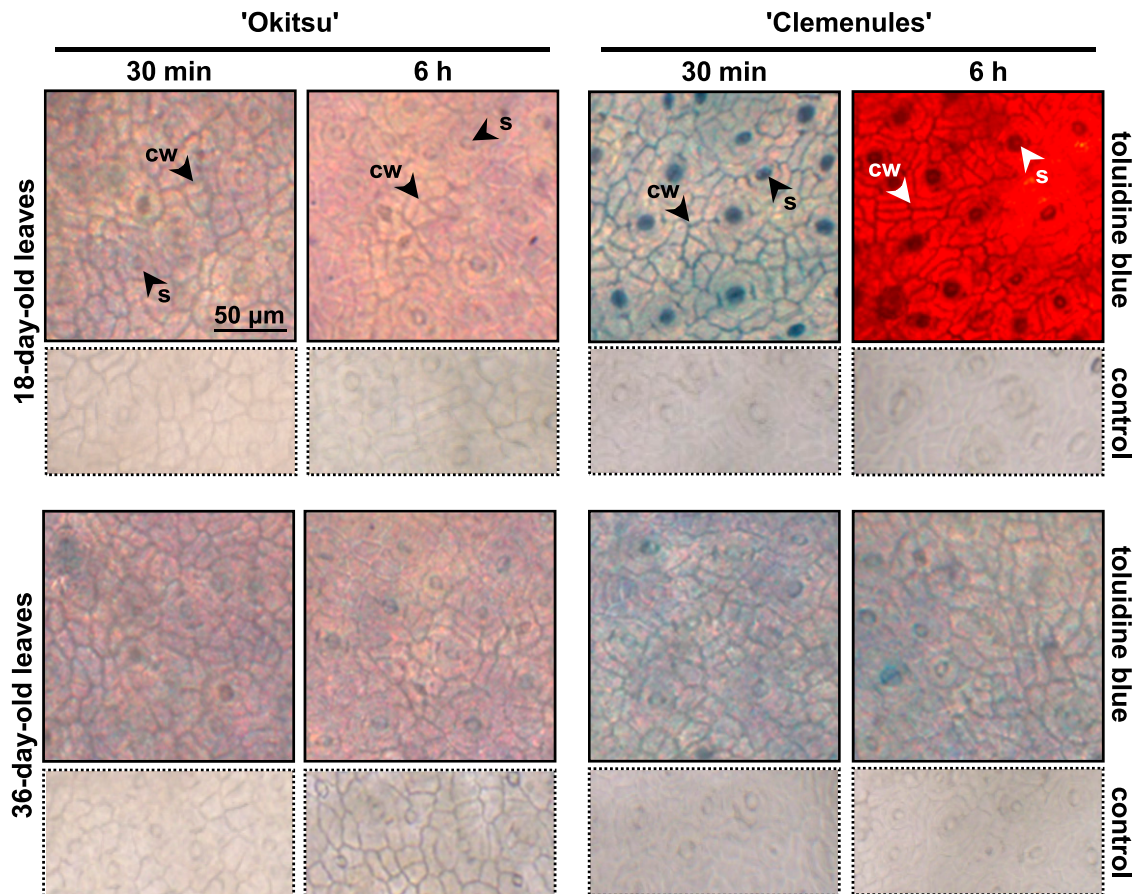

Fig. 2. Cuticle permeability is reduced in young 'Okitsu' leaves compared with 'Clemenules'. The permeability of the cuticle was assessed using toluidine blue and the leaves were photographed under white light. Permeability to the dye is visualized in 18-day-old 'Clemenules' epidermal tissue within the stomatal ledges, guard cells of stomata (s) and cell wall junctions (cw) after 30 min of exposure to the dye (blue staining), and in all subepidermal tissue after 6 h (red staining). Epidermal tissue of 18-day-old 'Okitsu' remained unstained. Control: leaves not treated with the dye. A minimum of 15 samples for each leaf age from three plants per mandarin cultivar were analyzed. Three independent experiments were conducted yielding similar results.

et al. 2008). To evaluate if there is a relationship between wax amount and resistance to *X. citri*, we studied the levels of total waxes in young and mature leaves from 'Okitsu' and 'Clemenules'.

In accordance with the increase of cuticular thickness, 18-day-old 'Okitsu' leaves showed significantly higher accumulation of total waxes per leaf unit area ($13.9 \pm 0.9\,\mu g\,cm^{-2}$) than 'Clemenules' ($3.7 \pm 0.3\,\mu g\,cm^{-2}$). Nevertheless, the chemical analysis of wax components of these leaf samples through GC-MS shows that wax profile shares high similarities between 'Okitsu' and 'Clemenules' (Table 2). The most abundant cuticular wax fraction in both mandarin cultivars was the primary alcohols, representing 96.6% in 'Okitsu' and 95.7% in 'Clemenules' of the GC-MS-detected compounds. They were accompanied by minor amounts of n-alkanes (2.4 and 3.2% for 'Okitsu' and 'Clemenules', respectively) and fatty acids (0.5 and 0.6% for 'Okitsu' and 'Clemenules', respectively). In the primary alcohol fraction, even-number homologs prevailed, such as hexacosanol (C_{26}), tetracosanol (C_{24}) and octacosanol (C_{28}). The second group was integrated by alkanes between C_{22} and C_{31}, such as docosano (C_{22}), tricosano (C_{23}) untriacontano (C_{31}), heptacosano (C_{27}), nonacosano (C_{29}) and pentacosano (C_{25}). Tetracosanoic (C_{24}), octadecanoic (C_{18}), hexadecanoic (C_{16}), and docosanoic (C_{22}) fatty acids established the third group in abundance of aliphatic lipids. Considering the differences between the total amounts of waxes per leaf unit area among the mandarin cultivars, the quantity of all these compounds was higher in 'Okitsu' than in 'Clemenules'. On the other hand, in 36-day-old leaves, the wax levels increased notably in 'Clemenules' ($16.4 \pm 0.5\,\mu g\,cm^{-2}$), reaching similar accumulation than in 'Okitsu' ($18.2 \pm 0.2\,\mu g\,cm^{-2}$). In this developmental stage, the predominant compounds were similar to those found in 18-day-old cuticle leaves and new compounds corresponding to aliphatic lipids of longer carbon chains were present in trace amounts in both cultivars, suggesting changes in the wax composition during leaf development.

The higher accumulation of waxes in 18-day-old 'Okitsu' leaf in comparison with 'Clemenules', suggests differences in expression of wax biosynthesis pathways between both mandarin cultivars. To further investigate this, the expression of candidate genes related to cuticular wax-biosynthesis was analyzed (Table 1; Fig. 4). Remarkably, the relative expression of *CER6*, a β-ketoacyl- CoA synthase (KCS), key in catalyzing the first step in VLCFAs formation was 4.6-fold higher in young 'Okitsu' leaves than in 'Clemenules'. Furthermore, the expression of *CER1* and *CER3*, which are involved in VLC alkane synthesis, was increased in the resistant cultivar compared with 'Clemenules' (induced by 5-fold and 4.5-fold, respectively). In the same way, the level of expression of *WIN1*, a transcription factor that regulates the expression of *CER1*, and *CER7*, a regulatory transcription factor of *CER3* (Hooker et al. 2007), was also elevated in the resistant cultivar (induced by 2.6-fold and 1.8-fold, respectively). Interestingly, the expression levels of VLC alcohol-forming genes, such as *CER4* and *FAR2* were higher in young 'Clemenules' leaves than in 'Okitsu'.

Taken together, these data show that resistance to *X. citri* correlates with a higher wax deposition, in accordance with the thicker cuticle shown in TEM analysis rather than differences in chemical cuticular composition.

Mechanical alteration of 'Okitsu' cuticle thickness allows *X. citri* colonization and canker development. To examine the hypothesis that the inhibition of bacterial survival on young 'Okitsu' leaf surface might be due to a higher wax accumulation, we analyzed the effect of reducing abaxial cuticle thickness on disease development.
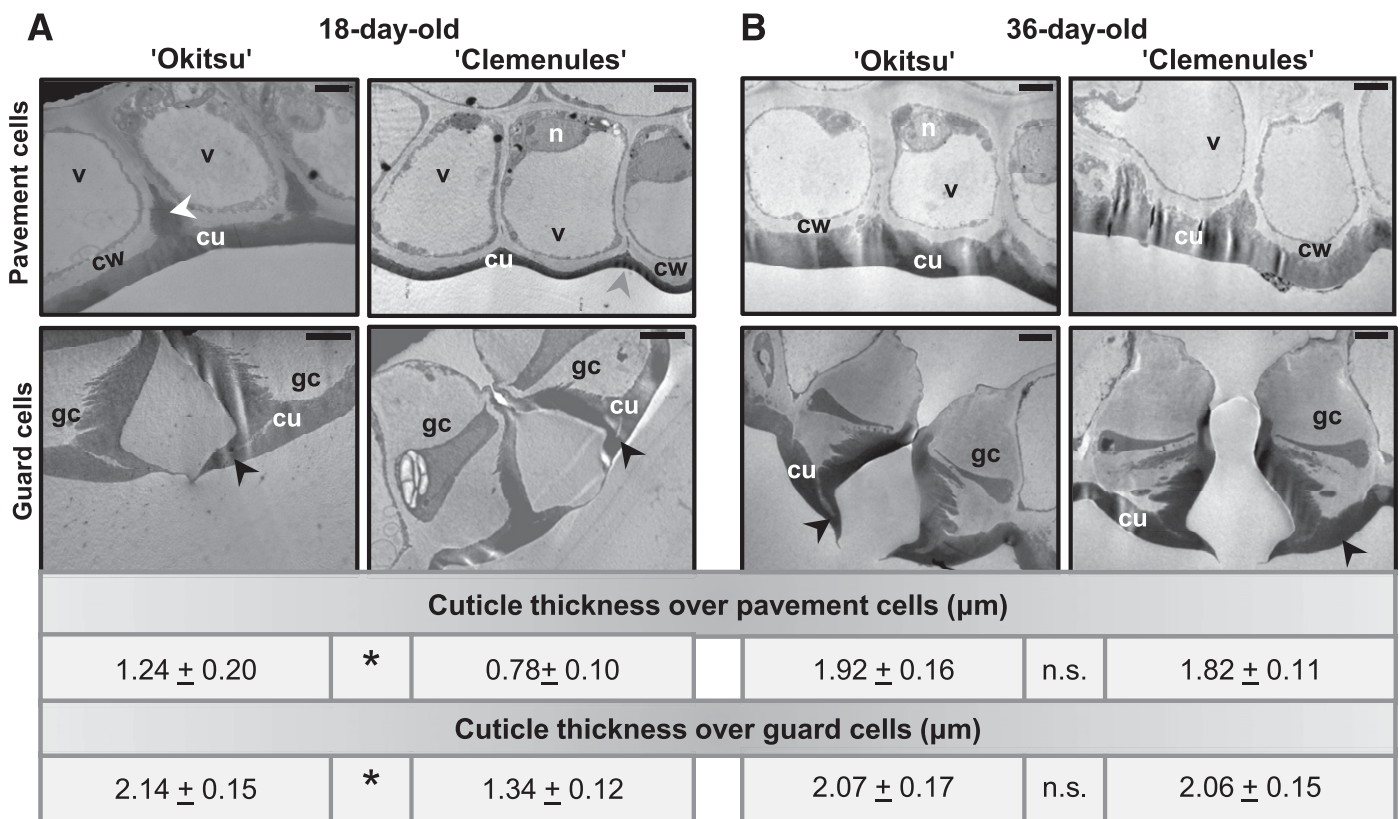

Cuticle thickness over pavement cells (µm)					
1.24 ± 0.20	*	0.78 ± 0.10	1.92 ± 0.16	n.s.	1.82 ± 0.11

Cuticle thickness over guard cells (µm)					
2.14 ± 0.15	*	1.34 ± 0.12	2.07 ± 0.17	n.s.	2.06 ± 0.15

Fig. 3. Epidermal structure in 'Okitsu' and 'Clemenules' leaves under transmission electron microscopy. Cuticle of abaxial epidermis of **A,** 18-day-old leaves and **B,** 36-day-old leaves. The thickness of the leaf cuticle (cu) and the stomatal cuticular edges are indicated by black arrows, and the cuticularization of anticlinal cell walls (cw) and the depressions of cell junctions are indicated by white and gray arrows, respectively. Scale bar = 2 µm. gc, guard cells; n, nucleus; and v, vacuole. The table indicates the measurement of the cuticle thickness in micrometers (µm). A minimum of six images were analyzed per leaf section. Three different leaves, obtained from different plants, were observed per genotype and age leaf. Similar results were observed in three separate experiments. Datasets marked with an asterisk are significantly different as assessed by Student's *t* test ($P < 0.05$). n.s. indicates not significant.

Epicuticular waxes from the leaf surface were selectively removed without damage to the epidermal cells using either cotton swabs or gum arabic (Gniwotta et al. 2005; Marcell and Beattie 2002).

Ultrastructural analysis showed that gum arabic and cotton swab treatments reduced cuticular thickness in epidermal pavement and guard cells from 18-day-old 'Okitsu' leaves. Cuticular thicknesses were 0.29 ± 0.09 µm and 0.18 ± 0.08 µm, for gum arabic and cotton swab treatments, respectively, compared with 1.24 µm for untreated leaves (Fig. 5A). Untreated 18-day-old 'Okitsu' and 'Clemenules' leaves were used as controls. Both treated and untreated leaves were inoculated by spraying with X. citri. The integrity of the epidermal layer and mesophyll tissue in untreated 'Okitsu' leaves remained unaltered after bacterial inoculation (Fig. 5B). The population size of X. citri began to decline 3 dpi and no canker symptoms were observed after 20 days, as expected from previous work (Favaro et al. 2014) (Fig. 5C). Notably, at 15 dpi, surface-treated 'Okitsu' leaves, whose epicuticular waxes were removed, showed the presence of bacteria invading the intercellular space of a hypertrophied mesophyll tissue. Moreover, these samples showed similar ultrastructural changes by bacterial colonization to 18-day-old 'Clemenules' leaves (Fig. 5B). Similar bacterial growth was observed between the surface-treated 'Okitsu' and untreated 'Clemenules' leaves. In these tissue samples, X. citri population gradually increased more than two orders of magnitude over the monitoring period that was correlated with the canker symptoms developed at 20 dpi (Fig. 5C).

These results confirm that 'Okitsu' cuticle interferes with X. citri fitness affecting early events required for bacterial infection and consequently mesophyll colonization.

Cuticular waxes inhibit X. citri growth. In order to determine if cuticular waxes from young 'Okitsu' leaves also act as an inhibitor of pathogen survival, we performed in vitro antibacterial activity assays. In an attempt to reproduce the
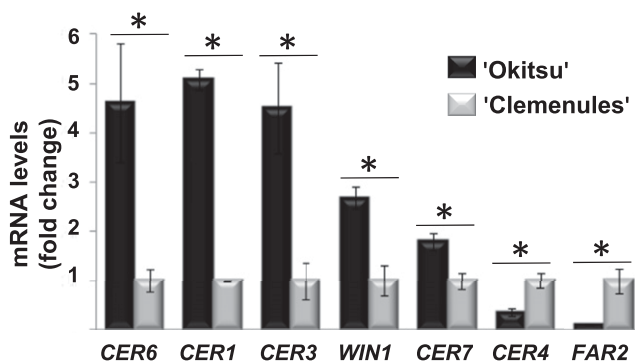

Fig. 4. Expression analysis of genes involved in cuticular wax biosynthesis. Quantitative polymerase chain reaction analysis of β-ketoacyl-CoA synthase KCS6/eceriferum 6 (CER6), eceriferum 1 (CER1), eceriferum 3 (CER3), wax inducer 1 transcription factor (WIN1), eceriferum 7 (CER7), fatty acyl-CoA reductase (CER4), and fatty acyl-CoA reductase (FAR2) mRNAs measured in 18-day-old leaves. The relative gene expression (ΔΔCt) fold change was performed considering 'Clemenules' leaves as reference samples and a histone H4 transcript as an endogenous control. Values are expressed as means ± standard deviation from three independent assays. Datasets marked with an asterisk are significantly different as assessed by Student's t test ($P < 0.05$).

TABLE 2. Cuticular waxes and their composition in 'Clemenules' and 'Okitsu' leaves[a]

	Wax composition (%)			
	18-day-old leaves		36-day-old leaves	
	'Okitsu'	'Clemenules'	'Okitsu'	'Clemenules'
Wax coverage (µg cm^{-2})	13.90	3.70*	18.20	16.40 n.s.
Primary alcohols				
Hexacosanol (C$_{26}$)	58.55 ± 0.055	49.38 ± 0.031	49.34 ± 0.729	40.10 ± 0.817
Tetracosanol (C$_{24}$)	23.94 ± 0.054	32.50 ± 0.021	21.65 ± 0.094	27.45 ± 0.128
Octacosanol (C$_{28}$)	9.36 ± 0.045	8.51 ± 0.046	10.86 ± 0.024	9.72 ± 0.047
Pentacosanol (C$_{25}$)	1.94 ± 0.007	2.18 ± 0.034	2.28 ± 0.076	2.62 ± 0.073
Heptacosanol (C$_{27}$)	1.26 ± 0.010	1.16 ± 0.050	1.69 ± 0.056	1.83 ± 0.078
Docosanol (C$_{22}$)	0.75 ± 0.000	1.40 ± 0.520	1.01 ± 0.087	1.79 ± 0.178
Triacontanol (C$_{30}$)	0.60 ± 0.038	0.18 ± 0.042	3.09 ± 0.059	1.36 ± 0.066
Tricosanol (C$_{23}$)	0.11 ± 0.029	0.24 ± 0.129	0.17 ± 0.022	0.36 ± 0.057
Nonacosanol (C$_{29}$)	0.06 ± 0.008	0.04 ± 0.066	0.30 ± 0.036	0.17 ± 0.033
Dotriacontanol (C$_{32}$)	0.04 ± 0.005	0.13 ± 0.007	3.01 ± 0.179	1.87 ± 0.131
Untriacontanol (C$_{31}$)	0.01 ± 0.000	0.02 ± 0.047	0.19 ± 0.024	0.09 ± 0.035
Tritriacontanol (C$_{33}$)	–	–	0.07 ± 0.011	0.04 ± 0.008
Tetracontanol (C$_{34}$)	–	–	0.04 ± 0.036	0.06 ± 0.031
Hexatriacontanol (C$_{36}$)	–	–	0.01 ± 0.005	–
Alkanes				
Untriacontano (C$_{31}$)	0.54 ± 0.008	1.03 ± 0.066	2.27 ± 0.016	4.86 ± 0.038
Pentacosano (C$_{25}$)	0.52 ± 0.000	0.52 ± 0.256	–	0.02 ± 0.000
Heptacosano (C$_{27}$)	0.35 ± 0.021	0.57 ± 0.232	0.23 ± 0.007	0.44 ± 0.023
Docosano (C$_{22}$)	0.35 ± 0.144	0.46 ± 0.399	–	–
Nonacosano (C$_{29}$)	0.32 ± 0.014	0.18 ± 0.114	0.69 ± 0.034	0.59 ± 0.037
Tricosano (C$_{23}$)	0.30 ± 0.003	0.11 ± 0.010	0.12 ± 0.000	1.69 ± 0.000
Octacosano (C$_{28}$)	–	–	0.05 ± 0.007	0.08 ± 0.003
Dotriacontano (C$_{32}$)	–	–	0.10 ± 0.011	0.60 ± 0.018
Tetratriacontano (C$_{34}$)	–	0.20 ± 0.025	0.16 ± 0.015	1.95 ± 0.051
Tetracosano (C$_{24}$)	–	0.26 ± 0.111	0.01 ± 0.000	0.01 ± 0.000
Fatty acids				
Tetracosanoic acid (C$_{24}$)	0.24 ± 0.016	0.36 ± 0.005	0.53 ± 0.055	0.50 ± 0.058
Octadecanoic acid (C$_{18}$)	0.10 ± 0.061	0.13 ± 0.000	0.68 ± 0.123	1.68 ± 0.123
Hexadecanoic acid (C$_{16}$)	0.06 ± 0.018	0.13 ± 0.000	0.43 ± 0.062	1.07 ± 0.048
Docosanoic acid (C$_{22}$)	0.06 ± 0.005	–	0.09 ± 0.010	0.05 ± 0.006
Hexacosanoic acid (C$_{26}$)	0.11 ± 0.000	0.05 ± 0.002	0.26 ± 0.015	0.20 ± 0.017

[a] Quantification and composition of cuticular waxes per unit of leaf area of both cultivars and in different development stages are expressed as means from three independent experiments. The dataset marked with an asterisk is significantly different as assessed by Student's t test ($P < 0.05$). n.s. indicates not significant.
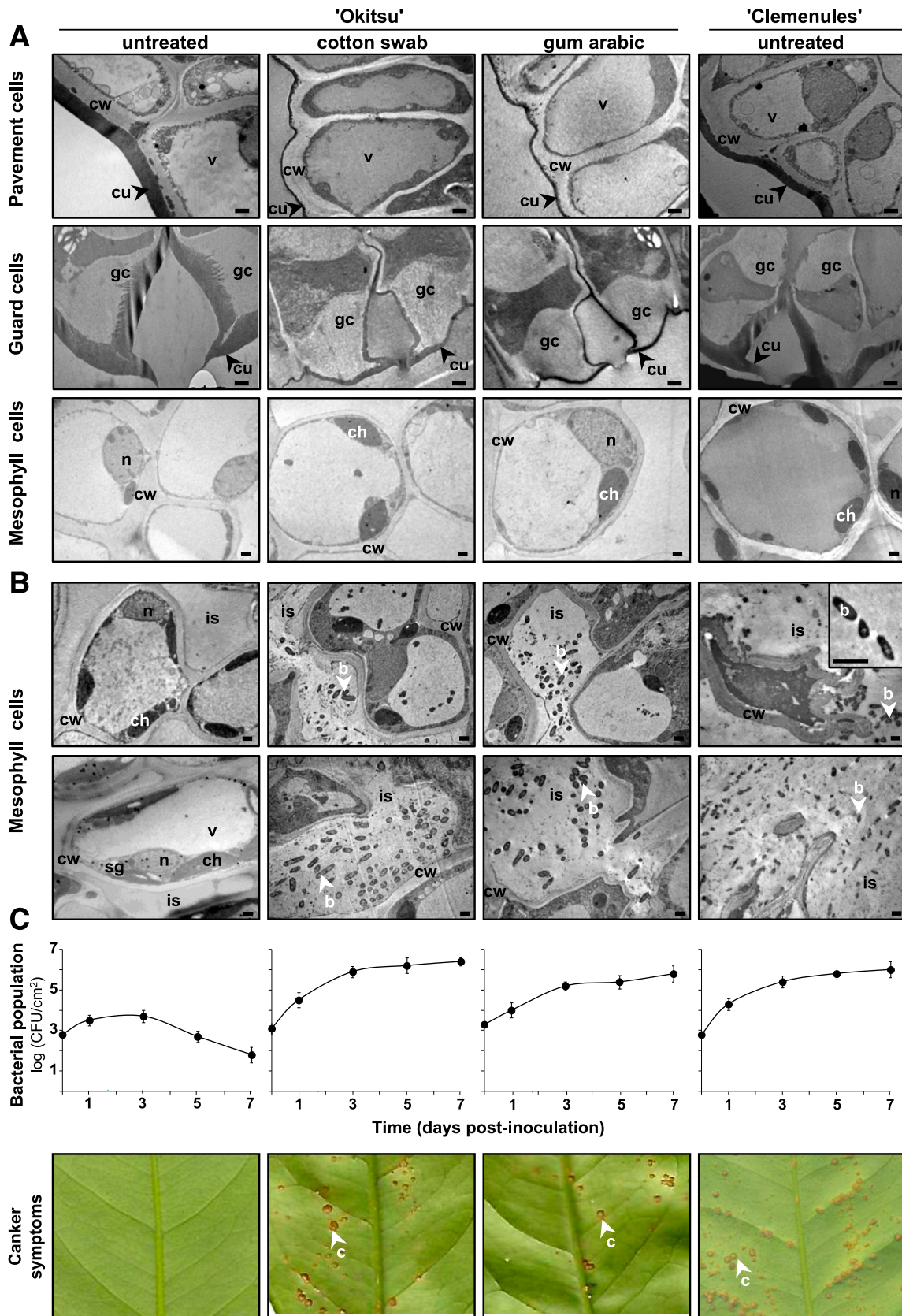
Fig. 5. Ultrastructural and phenotypic features of young 'Okitsu' leaves inoculated with *Xanthomonas citri* after removal of epicuticular wax. **A,** Overview of uninfected leaf tissue showing cuticle (cu) structure of pavement cells, guard cells (gc), and mesophyll tissue before and after mechanical removal of 'Okitsu' epicuticular waxes. Differences in cuticle thickness between leaves treated with cotton swab or gum arabic and untreated leaf (black arrow). Scale bar = 1 μm. **B,** Ultramorphological changes of *X. citri*-infected tissues 15 days postinoculation (dpi). Scale bar = 1 μm. b, bacteria; cw, cell wall; ch, chloroplast; n, nucleus; is, intercellular space; sg, starch granule; and v, vacuole. **C,** In vivo growth of *X. citri* on treated mandarin leaves, whose epicuticular waxes were extracted, and untreated mandarin leaves. Values are expressed as means ± standard deviation of triplicate measurements from three independent experiments. Bacterial populations in untreated 'Okitsu' leaves significantly differed from those found in 'Clemenules' and treated 'Okitsu' leaves from 3 to 7 dpi (one-way analysis of variance, Tukey's test, $P <$ 0.05). Symptom development induced by *X. citri* strain on lower surfaces of treated and untreated mandarin leaves 20 dpi. c, canker lesions.

conditions in planta, *X. citri* was exposed to the cuticular waxes on solid medium. Cuticular waxes extracted from both 18-day-old young 'Okitsu' and 'Clemenules' leaves have a potent inhibitory effect on the growth of *X. citri* compared with the control (Fig. 6A). Notwithstanding, 'Okitsu' waxes have an antibacterial activity 50% higher than 'Clemenules', according to the level of cuticular waxes per leaf unit area (Fig. 6A). In order to follow the waxes effect on the bacterial growth over a 12 h time course, the cells were propagated in liquid medium supplemented with 'Okitsu' or 'Clemenules' cuticular waxes. This study confirmed that the higher amount of cuticular waxes per square centimeter of leaf area in 'Okitsu' compared with 'Clemenules' results in a greater antibacterial activity in the resistant cultivar (Fig. 6B). These results indicate that the increase of wax accumulation play a role in the resistance against *X. citri*, contributing not only as preformed defenses but also as an antimicrobial agent.

DISCUSSION

The plant leaf surface, or the phyllosphere, is one of the most important natural habitats for microorganisms. It has been demonstrated that epiphytic bacterial populations are directly influenced by certain environmental conditions of the phyllosphere such as fluctuating temperature, radiation, relative humidity, presence of free water, and the availability of plant-leached metabolites at the leaf surface, so that only adapted bacteria can survive (Aragón et al. 2017; Schlechter et al. 2019; Schreiber et al. 2005; Whipps et al. 2008). In previous studies, we showed that in young 'Okitsu' leaves surface defense barrier impedes epiphytic growth and biofilm formation of *X. citri*, conferring resistance to canker disease (Favaro et al. 2014). In this work, we investigated the physical and chemical characteristics of this defense barrier.

Changes in stomata aperture size are function of both guard and epidermal cell turgor, regulated by signaling components in guard cells in response to environmental conditions, including abiotic and biotic stress (Melotto et al. 2017). Our results indicate that a thicker cuticle is correlated with a smaller stomatal aperture and less permeable stomatal cuticular edges. *Arabidopsis* mutants that are unable to synthesize cutin have diminished cuticular projections surrounding the stomatal pore resulting in increased susceptibility to pathogens (Li et al. 2007). Considering the range of stomatal aperture in young 'Okitsu' leaves (0.3 to 0.6 µM) and the *X. citri* size (1.5 to 2.0 × 0.5 to 0.75 µm) (Goto 1992), we could hypothesize that the stomatal cuticular edges enhance stomatal defense by reducing bacterial entry into the mesophyllic tissue. The absence of both bacterial proliferation and hypertrophy in the mesophyll tissue after bacterial inoculation of 'Okitsu' young leaves support this hypothesis.

We demonstrated that 'Okitsu' cuticle in young leaf is almost twofold thicker compared with 'Clemenules'. No differences in cuticle thickness between cultivars were observed in mature (36-day-old) leaves. This result indicates that 'Okitsu' cuticle develops rapidly during the leaf expansion process, which is the period of optimal susceptibility to *X. citri* infection. A thicker 'Okitsu' cuticle was related with the fortification of epidermal anticlinal cell wall, which generates a smooth surface, whereas the leaf cuticle has a sinuous or rough surface in 'Clemenules'. It has been demonstrated that these cavities formed in epidermal cell wall junctions are protected sites where phytopathogenic bacteria survive (Lindow and Brandl 2003; Schlechter et al. 2019; Whipps et al. 2008). Furthermore, we found a negative relationship between the cuticular thickness and water permeability on 'Okitsu' leaf, which may be associated with a low availability of water and nutrients on leaf surface, preventing also the epiphytic growth of *X. citri*. It has been extensively demonstrated that changes in cuticle permeability influence plant–bacterial interactions (Aragón et al. 2017; Tang et al. 2007; Xiao et al. 2004; Yeats and Rose 2013; Ziv et al. 2018). Moreover, a number of epiphytic (pathogenic and nonpathogenic) bacteria have been shown to increase cuticular permeability,

enhancing solubilization and diffusion of nutrient from the leaf to improve epiphytic fitness on the leaf surface (Lindow and Brandl 2003; Schreiber et al. 2005; Vacher et al. 2016; Whipps et al. 2008).

The cuticular thickness of 'Okitsu' and its consequence over leaf permeability could be explained considering a greater quantity of total cuticular waxes. In young 'Okitsu' leaves, the higher expression of genes involved in the first step of VLCFA formation, coupled with the up regulation of genes involved in VLC alkane production, resulted in a higher accumulation of wax constituents, may indicate that the wax biosynthetic pathways are induced earlier in this resistant cultivar compared with 'Clemenules' in the same phenological stage. Although primary alcohols dominated the wax mixture in leaves of both mandarin cultivars, the absolute amounts of these compounds (referred as µg cm^{-2} of the leaf) differ between them. We could speculate that the higher expression levels of VLC alcohol-forming genes, such as *CER4* and *FAR2* in young 'Clemenules' leaves might mean an enhanced synthesis, to reach similar levels to 'Okitsu' in
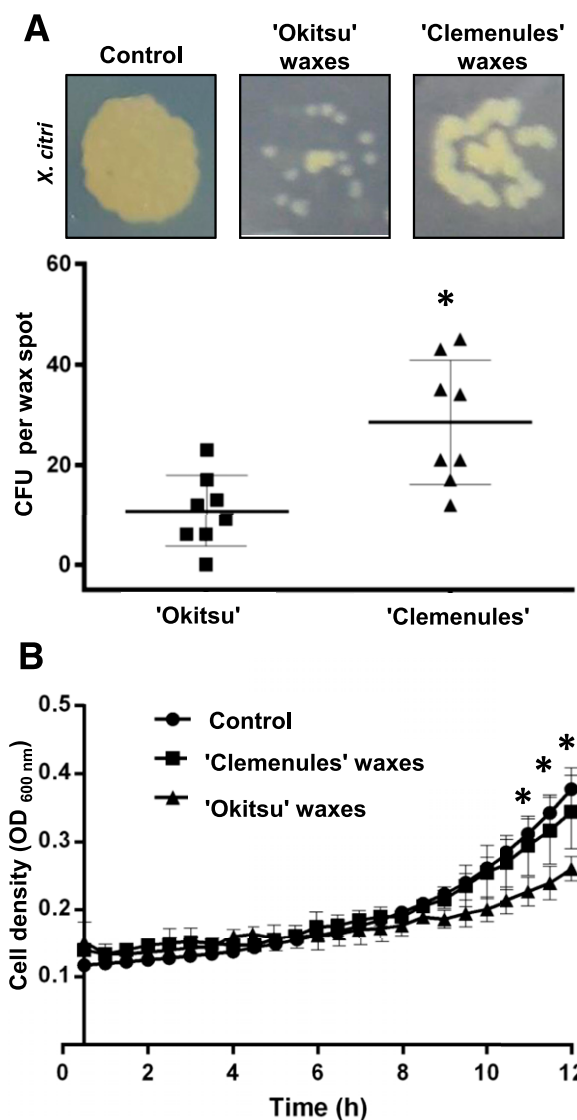

Fig. 6. Antimicrobial activity of cuticular wax extracts isolated from young 'Okitsu' and 'Clemenules' leaves against *Xanthomonas citri*. **A,** Growth of *X. citri* over cuticular wax and control spots in NYG medium (peptone extract at 5 g liter^{-1}, yeast extract at 3 g liter^{-1}, and glycerol at 20 g liter^{-1}) agar plate. The graph shows the number of bacterial colonies per mandarin cuticular wax spot. **B,** *X. citri* growth in NYG broth supplemented with cuticular wax extract. Values are expressed as means ± standard deviation of three independent experiments. Datasets marked with an asterisk are significantly different as assessed by Student's *t* test ($P < 0.05$).

mature leaves. Riederer and Schneider (1990) also found a higher quantity of primary alcohols in *C. aurantium* as leaves age increased. The increase of cuticular wax production during leaf development has been also reported in other plant species (Lee and Suh 2015; Yeats and Rose 2013; Zhu et al. 2018).

It has been proposed that waxy broad-leaved plants support lower populations of culturable bacteria in the phyllosphere due to avoidance of water stagnation on the plant surface (Marcell and Beattie 2002; Whipps et al. 2008). Thus, the abundance of epicuticular waxes has been associated to a self-cleaning mechanism known as the lotus effect, which repels water avoiding pathogen establishment (Yeats and Rose 2013). Hydrophobicity of the epicuticular waxes depends on the nature of the chemical groups exposed on the surface (Marcell and Beattie 2002). Although there are no significant differences in cuticular wax compositions between 'Okitsu' and 'Clemenules', the larger amount of cuticular waxes in young 'Okitsu' leaves could be associated with a greater hydrophobicity compared with 'Clemenules', and consequently, with a greater water-repellent surface, which interferes with the proliferation of *X. citri* in the epiphytic phase. Glossy maize mutants (*gl1*, *gl3*, and *gl5/gl20*), which produce less epicuticular waxes and have a less hydrophobic surface than wild-type, support greater epiphytic growth of the pathogenic bacteria *Clavibacter michiganensis*, presumably due to the increased leaching of nutrients from mesophyll (Marcell and Beattie 2002). According to our results, the mechanical removal of epicuticular waxes of the abaxial surface of young 'Okitsu' leaves make it susceptible to *X. citri* infection, indicating that waxes are involved in the resistance mechanism. In accordance with our results, a high association between epicuticular wax content and resistance to *X. citri* was shown in *Fortunella* 'Meiwa' (Wang et al. 2011).

Over recent years, significant progress has been made to understand the biological role of cuticular waxes in the susceptibility or resistance to fungal infection (Aragón et al. 2017; Batista dos Santos et al. 2019; Hansjakob et al. 2010). However, there is no data about the activity of the *Citrus* leaf cuticular waxes against plant bacterial pathogens. Here, we show that cuticular waxes of both mandarin cultivars have antibacterial activity in vitro against *X. citri*. Nevertheless, cuticular waxes from young 'Okitsu' leaves have a greater inhibitor effect than 'Clemenules' at the same developmental stage, which is correlated with its higher amount of waxes. The most abundant wax components in both cultivars are primary alcohols, whose mix is known as policosanol. Further research is necessary to understand the importance of these compounds as antibacterial agents.

In conclusion, we provide evidence for the 'Okitsu' resistance to *X. citri* infection by physiological, biochemical, and ultrastructural analysis of its cuticle. The presence of a higher amount of cuticular waxes, particularly epicuticular ones, in the beginning of 'Okitsu' leaf development would lead to less susceptibility to *X. citri* infection in this genotype. Plant cuticle reinforcement in young leaves could then be used as a functional trait to manage foliar bacterial diseases.

ACKNOWLEDGMENTS

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