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Novel demonstration of RNAi in citrus reveals importance of citrus callose synthase in defence against *Xanthomonas citri* subsp. *citri*

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Summary

Citrus is an economically important fruit crop that is severely afflicted by citrus canker, a disease caused by the bacterial phytopathogen, *Xanthomonas citri* subsp. *citri* (*Xcc*). GenBank houses a large collection of Expressed Sequence Tags (ESTs) enriched with transcripts generated during the defence response against this pathogen; however, there are currently no strategies in citrus to assess the function of candidate genes. This has greatly limited research as defence signalling genes are often involved in multiple pathways. In this study, we demonstrate the efficacy of RNA interference (RNAi) as a functional genomics tool to assess the function of candidate genes involved in the defence response of *Citrus limon* against the citrus canker pathogen. Double-stranded RNA expression vectors, encoding hairpin RNAs for citrus host genes, were delivered to lemon leaves by transient infiltration with transformed *Agrobacterium*. As proof of principle, we have established silencing of citrus phytoene desaturase (*PDS)* and callose synthase (*CalS1*) genes. Phenotypic and molecular analyses showed that silencing vectors were functional not only in lemon plants but also in other species of the *Rutaceae* family. Using silencing of *CalS1*, we have demonstrated that plant cell wall-associated defence is the principal initial barrier against *Xanthomonas* infection in citrus plants. Additionally, we present here results that suggest that H_2O_2 accumulation, which is suppressed by xanthan from *Xcc* during pathogenesis, contributes to inhibition of xanthan-deficient *Xcc* mutant growth either in wild-type or *CalS1*-silenced plants. With this work, we have demonstrated that high-throughput reverse genetic analysis is feasible in citrus.

Keywords: RNAi-induced gene silencing, citrus functional genomics, *Xanthomonas citri* subsp. *citri,* asiatic citrus canker, callose synthase.

Introduction

Citrus is the most economically significant fruit tree crop in the world, with an annual production of 105 million tons. Lemon, classified as *Citrus limon* (L.) Burm. f. (Tanaka, 1969) is the third most important *Citrus* species after orange and mandarin. The Tucumán Province of Argentina is one of the world's largest lemon producers, with an annual output of approximately 1.47 million tons. A high percentage (60%) of its production is designated for industry processing (elaboration of concentrated juice, essential oils and dried lemon peels) and the rest is commercialized as fresh fruit (http://www.fas.usda.gov/currwmt.asp/Citrus: World Markets and Trade). Production satisfies domestic fresh lemon fruit demand throughout the year and generates a surplus that is sold to meet the off-season demands of markets in the northern hemisphere.

Asiatic citrus canker is caused by *Xanthomonas axonopodis* pv. *citri (Xac)*, recently renamed *Xanthomonas citri*

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subsp. *citri* (*Xcc*) (Schaad *et al.*, 2006), which is the most severe and wide-spread bacterial pathogen to afflict citrus (Graham *et al.*, 2004). Most of the world's commercial citrus cultivars are moderately to highly susceptible to *Xcc*, and once established, the pathogen is difficult to eradicate. Quarantine is used to prevent spread to new geographic regions. Disease management options are currently limited to the use of copper-based bactericides, destruction of diseased trees and surrounding areas, and replacement with less susceptible cultivars where possible (Graham *et al.*, 2004).

There is great need for new and sustainable strategies to manage asiatic citrus canker. Field evaluations suggest that full or partial resistance to *Xcc* does exist among several types of citrus and closely related genera, including *Citrus ichangensis*, *C. junos*, *C. medica*, *C. unshiu*, *Citrofortunella* and *Fortunella spp* (Schubert *et al.*, 2001; Lee *et al.*, 2009; Shiotani *et al.*, 2009). However, characterization of this resistance has only recently begun (Khalaf *et al.*, 2007; Lee *et al.*, 2009; Shiotani *et al.*, 2009). The other option, cultivar improvement through traditional breeding continues to be a difficult process, obstructed by apomixes, sexual incompatibility, prolonged juvenility, high heterozygosity and the complex hybrid nature of citrus. In fact, the citrus cultivars with the greatest worldwide economic significance (e.g. *C. sinensis*, *C. paradisi*, and *C. limon*) are not biologically defined species, but are rather the result of accumulated somatic mutation or natural hybridization in the wild places or field (Gmitter, 1995).

Biotechnology offers an alternate strategy for sustainable management of asiatic citrus canker, and citrus genomics is providing new tools for crop improvement. A large database of Expressed Sequence Tags (~559 000 ESTs) has recently been generated from a variety of *Citrus* species (International Citrus Genome/Genomics Consortium, http://www.citrusgenome.ucr.edu/), covering a wide range of tissues, developmental stages, and abiotic and biotic stress conditions (Forment *et al.*, 2005; Targon *et al.*, 2007; Terol *et al.*, 2007; Martinez-Godoy *et al.*, 2008; Marques *et al.*, 2009). In citrus, gene expression associated with pathogen response has been analysed in fungal (leaf spot, *Alternaria*; green mould, *Penicillium digitatum*; postbloom fruit drop, *Colletotrichum acutatum*; root rot, *Phytophthora parasitica*), bacterial (canker, *Xanthomonas* spp.; citrus variegated chlorosis, *Xylella fastidiosa*) and viral diseases [citrus tristeza virus (CTV); citrus leprosis virus (CiLV)] (Lahey *et al.*, 2004; Marcos *et al.*, 2005; Campos *et al.*, 2007; Cristofani-Yaly *et al.*, 2007; Freitas-Astua *et al.*, 2007; Gandia *et al.*, 2007; Khalaf *et al.*, 2007; Souza *et al.*, 2007a,b; Targon *et al.*, 2007; Cernadas *et al.*, 2008).

Although candidate genes for citrus improvement have been identified and selected from these data, a significant number of unigenes (~15–20%) are unique to *Citrus* species and their biological functions have yet to be determined (Forment *et al.*, 2005; Martinez-Godoy *et al.*, 2008). RNA interference (RNAi) of plant genes, initiated by the delivery of double-stranded RNA (dsRNA) (Wesley *et al.*, 2001; Helliwell and Waterhouse, 2003), is an attractive forward and reverse genetic tool for the study of gene function in polyploidy species as citrus plants. In this work, we have tested the use of RNA silencing in *C. limon* plants based on RNAi technology, using an intron-containing self-complementary 'hairpin' RNA (hpRNAi) construct (Helliwell and Waterhouse, 2003). To induce silencing, dsRNAs were delivered by infiltrating lemon leaves with *Agrobacterium tumefaciens* carrying the hpRNAi-*transgene*. Two target genes have been analysed: citrus phytoene desaturase (*PDS*) and citrus callose synthase (*CalS*) (Verma and Hong, 2001), also referred as glucan synthase-like (*GSL*) (Richmond and Somerville, 2000). The *PDS* gene was selected as a control. Its reduction confers a clear photobleaching phenotype on the leaves, and it has been used successfully as a control in several RNA silencing systems (Kumagai *et al.*, 1995; Ruiz *et al.*, 1998; Liu *et al.*, 2002; Brigneti *et al.*, 2004).

Callose is the predominant cell wall polysaccharide, consisting of β-1,3-glucan chains, present in the nascent cell plate during normal plant growth and development (Samuels *et al.*, 1995; Thiele *et al.*, 2009) and is also involved in response to biotic and abiotic stress (for a recent review see Chen and Kim, 2009). Callose is synthesized by callose synthases, which are membrane-bound enzymes that have been relatively well characterized in *Arabidopsis* (Chen and Kim, 2009). Most information on callose in plants comes from analysis of *CalS/GSL* knockout mutations in *Arabidopsis*, which has 12 putative *CalS/GSL* genes (Vogel and Somerville, 2000; Jacobs *et al.*, 2003; Nishimura *et al.*, 2003; Dong *et al.*, 2008; Thiele *et al.*, 2009). It has been suggested that multiple *CalS* genes may have evolved in higher plants for the synthesis of callose in different locations and in response to different physiological and developmental signals (Verma and Hong, 2001; Nishimura *et al.*, 2003; Enns *et al.*, 2005; Thiele *et al.*, 2009). The regulation of *CalS* genes expression during plant development and after pathogen infection has recently been reported in *Arabidopsis* (Dong *et al.*, 2008). Callose

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synthase was chosen as an RNAi silencing target in this study because callose plays a role in key plant processes, but the nature and function of *CalS* genes have yet to be fully characterized in citrus. The *C. limon CalS* sequence shares 83% identity with the *Arabidopsis CalS1/GSL6* catalytic subunit, which is located at the growing cell plate, interacts with cell plate-associated proteins (Hong *et al.*, 2001) and is induced in response to pathogen attack (Dong *et al.*, 2008). We hypothesized that the loss of *CalS1* function through RNAi silencing in citrus would produce altered leaf morphology. Given that previous work has indirectly implicated callose deposition in the resistance of host plants to infection by *Xanthomonas campestris* pv. *campestris* (Yun *et al.*, 2006), we decided to examine the influence of *CalS1* silencing on the outcome of this *Citrus-Xcc* interaction. Our results demonstrate that plant cell wall-associated defence is the principal initial barrier against *Xanthomonas* infection in citrus plants and that callose deposition and H_2O_2 accumulation in *C. limon*, which is suppressed by *Xanthomonas citri* subsp. *citri* during pathogenesis, contributes to inhibition of xanthan-deficient *Xcc gumB* mutant in both wild-type and *CalS1*-silenced plants.

Results

Testing the effectiveness of RNA silencing in *C. limon*

To examine whether hpRNA-derived siRNAs can be used to characterize gene function and manipulate the defence response to *Xanthomonas* in citrus plants, we first tested the efficiency and extent of RNA silencing in citrus using the endogenous lemon *PDS* gene. We chose this gene because loss of the PDS enzyme blocks carotenoid synthesis culminating in photo-oxidation of chlorophylls and a photobleaching phenotype that facilitates visual monitoring of silencing induction and progression (Kumagai *et al.*, 1995).

The *C. limon PDS* (*Cl-PDS*) sequence showed 100% identity with both orange *PDS* (gb/AY669082.1) and mandarin *PDS* (AB046992) genes. A binary hpRNA vector (pHG12 *Cl-PDS*) regulated by the 35S promoter was constructed from pHELLSGATE12 by replacing the *ccdB* coding region flanked by *att*L sites with the lemon *Cl-PDS* gene using Gateway technology (Figure 1a). The second pair of leaves of four- to six-leaf stage plants grown from seeds of *C. limon* cultivars Eureka Frost and Lisboa Frost were agro-infiltrated with two clones chosen at random, pHG12 C*l-PDS*-F and pHG12 C*l-PDS*-R (one construct per plant, allowing for four vector-variety combinations). A representative four- to six-leaf stage plant is shown in Figure 1b. At 15–25 days postinoculation (dpi), symptoms of *PDS* silencing resulting from local RNAi induction were observed only in inoculated tissues (Figure 1c). At 60 dpi, 90% of the twenty agro-infiltrated plants showed some degree of silencing-induced photobleaching. Within a single plant, a varied degree of *PDS* silencing (RNAi*PDS*) was observed in uninoculated systemic leaves. Initially silencing was limited to regions surrounding the main veins (Figure 1d). Later as new leaves formed, we observed spread of *PDS* silencing across the whole leaf, indicating that the silencing signal moves between cells and over long distances. Strong silencing in test plants resulted in leaves that were almost completely white (Figure 1d, f). The leaves of control wild-type plants of either cultivar remained green after infiltration with *Agrobacterium* EHA105 alone (Figure 1e).

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis showed that endogenous *PDS* mRNA levels were 64% lower in leaves showing strong photobleaching in pHG12 *Cl-PDS*-infiltrated plants compared to levels observed in control plants (data not shown).

We evaluated persistence of the phenotype for 6–12 months. Plants with a high degree of *PDS* silencing died after total photobleaching. Plants less severely affected recovered after losing photobleached leaves. Among the new growth, only some lateral leaves displayed photobleaching (Figure 1g). No clear difference in the silencing phenotype was observed among any of the four combinations of *PDS* vectors and citrus cultivars (data not shown).

Phenotype of the callose synthase silencing plants

We chose one putative EST sequence of the citrus callose synthase gene, *CalS/GSL*, from *C. reshni* (CX305437) to design primers to amplify a cDNA fragment of the *CalS/GSL* coding region of *C. limon* cv. Eureka. This EST sequence of *C. limon CalS1* (*Cl-CalS1*) shares homology with a number of *CalS/GSL* sequences from *Arapidopsis*. Among these, it most closely homologous with the published *Arabidopsis CalS1/GSL6* gene (AF237733) at 83% identity. In *Arabidopsis*, *CalS1* gene expression is regulated in a tissue-specific manner in leaves and root hairs and is induced by salicylic acid treatment and upon pathogen infection (Dong *et al.*, 2008).

cDNA was used to construct a hairpin RNAi vector targeted to silence the endogenous citrus *CalS1* gene

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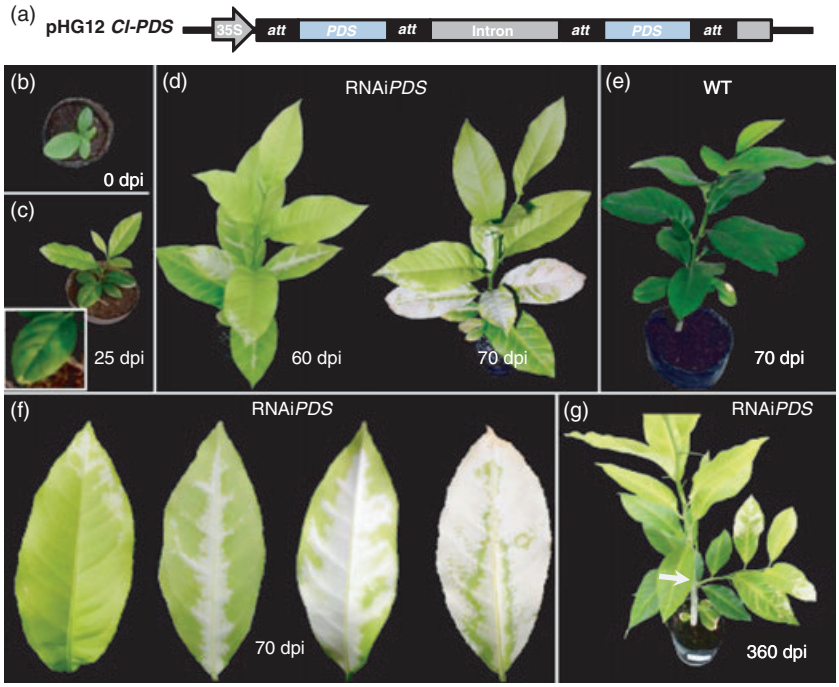

Figure 1 *Citrus limon* plants showing *PDS* silencing phenotype. (a) Schematic diagram of the pHG12 *Cl-PDS* plasmid construct; 35S, CaMV 35S promoter; att, Gateway ™ att sites (b) A plant at four-leaf stage agro-infiltrated with pHG12 *Cl-PDS*. (c) The same plant at 25 days postinoculation (dpi), the agro-infiltrated leaf shows a close up (framed) of a *PDS*-silenced sector. (d) The same plant at 60 and 70 dpi, respectively. (e) Wild-type (WT) plants agro-infiltrated with bacteria (no vector) at 70 dpi. (f) Magnification of systemic leaves from plant displayed in (d), showing distinct *PDS* silencing phenotypes. In the first, leaf bleaching begins at the distal end; in the other, leaf bleaching begins from the central vein and extends to the periphery of the leaf from the secondary veins. (g) After pruning, a new branch sprouted maintaining the bleached phenotype (white arrow). Three independent biological assays were carried for each of the two cultivars (*C. limon* cv. Eureka Frost and *C. limon* cv. Lisboa Frost) and yielded similar results. In each assay, twenty lemon seedlings were used per cultivar, of which half were infiltrated with the transformed silencing vector and half were infiltrated with agrobacteria alone.

(pHG12 *Cl-CalS*1). A suspension of *Agrobacterium* containing the pHG12 *Cl-CalS1* construct (Figure 2a) was infiltrated into the second pair of leaves of four- to six-leaf stage plants grown from seeds of *C. limon* cv. Eureka. Approximately 5–10 days post-treatment, only the leaves infiltrated with pHG12 *Cl-CalS1* developed a rough phenotype (Figure 2b). At 50 dpi, 100% of the 15 plants inoculated with the pHG12 *Cl-CalS1* construct were noticeably shorter in height than the control wild-type plants, which showed a normal phenotype after inoculation with untransformed *Agrobacterium* (Figure 2c). The internode length of *CalS1*-silenced (RNAi*CalS1*) plants was shorter compared to those in wild-type plants (0.72 ± 0.088 cm vs. 1.4 ± 0.48 cm); however, the internode width and total number of nodes on the main stem were roughly the same in both plants (Figure 2c). The leaves of silenced plants were smaller on average than wild-type leaves and had a wrinkled texture, bumps and a heart shape (Figure 2d). These plants maintained the same phenotype 1-year post-treatment, indicating high silencing stability during that period of time (Figure 2e). To determine whether the silencing phenotype could be propagated by grafting, RNAi*CalS1* plant scions were budded onto rootstocks of Troyer citrange (Figure 2f), another member of the *Rutacea* family. The propagation efficiency was >30%, and 80% of the regenerated shoot grafted shown the RNAi*CalS1* phenotype (Figure 2g).

To test whether citrus silencing methodology would work in other *Rutacea* species, we compared RNAi*CalS1* phenotypes on citrus and Troyer citrange seedling plants. The RNAi*CalS1* phenotypes were similar between the two *Rutacea* species. RNAi*CalS1* Troyer citrange plants were also markedly shorter in stature than the Troyer citrange control wild-type plants infiltrated with *Agrobacterium* EHA105 alone (Figure 2h), though the number of nodes was approximately the same. At 40–50 dpi, the new leaves from RNAi*CalS1* Troyer citrange plants were smaller than wild-type plants and appeared bilobular rather than trifoliar, which is characteristic Troyer citrange leaves (Figure 2h). We evaluated persistence of the phenotype

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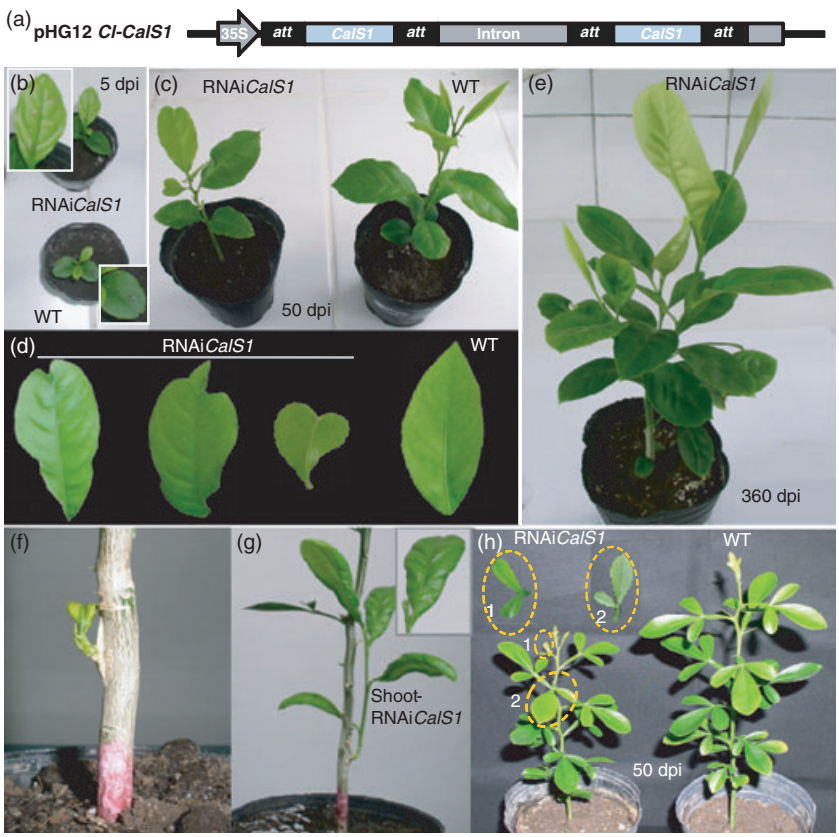

Figure 2 Phenotype of *CalS1* silencing in *Citrus limon* plants. (a) Schematic diagram of the pHG12 *Cl-CalS1* construct; 35S, CaMV 35S promoter; att, Gateway™ att sites (b) Induction of *CalS1* silencing in agro-infiltrated lemon leaf with pHG12 *Cl-CalS1* and wild-type (WT) plants agro-infiltrated with bacteria (no vector) at 5 days postinoculation (dpi). (c) The same RNAi*CalS1* silencing plants after 50 dpi, WT plants at the same stage. (d) Systemic leaves with distinct *CalS1* silencing phenotypes from a single plant at 50 dpi and compared with the WT leaves. (e) *CalS1* silencing in *C. limon* after 360 dpi. (f) RNAi*CalS1* lemon shoot grafted onto Troyer citrange rootstock at 20 days and (g) 5 months after budding, magnification of leaf phenotype (insert). (h) RNAi*CalS1* silencing in Troyer citrange after 50 dpi. Areas within the dotted yellow lines show a bilobular phenotype in RNAi*CalS1* leaves. Similar phenotypes were observed in 15 lemon and eight citrange plants analysed in at least three separate experiments.

for 15 months and 40% of the emerging leaves were bilobulates in the RNAi*CalS1* citrange plants (data not shown).

Histo-anatomic and molecular analysis of the RNAi*CalS1* lemon leaves

Leaf anatomy of RNAi*CalS1* lemon plants was analysed and compared with the leaf anatomy of wild-type plants. Wild-type leaves showed a highly organized structure (Figure 3a). The adaxial epidermis (AdE) was unilayered, regular in size, contained square shaped cells, and was covered by a thin and smooth cuticle. Dorsiventral mesophyll was differentiated into 2–3 layers of palisade parenchyma (PP) and several layers of spongy parenchyma (SP) cells, which were loosely arranged, with considerable intercellular spaces between them. The abaxial epidermis (AbE) was unstratified and contained square-shaped cells. Strikingly, the RNAi*CalS1* leaves showed notches at the epidermal level, and cells on both AdE and AbE were rectangular in shape, rather than square (Figure 3b). The mesophyll was disorganized; the PP cells appeared smaller than wild-type tissue cells and the spongy cells were tangentially flattened and more loosely arranged and dispersed than the wild-type plants.

We performed qRT-PCR to confirm suppression of endogenous *CalS1* mRNAs in *C. limon*, using accumulation of actin transcripts as an internal amplification control and for signal normalization. RNA samples from silenced and control plants were extracted at 60 dpi and amplified using primers specific to the target gene. Systemic leaves selected for the silenced sample, either from RNAi*CalS1* plants or regenerated RNAi*CalS1* shoot grafts, displayed strong phenotypic symptoms of callose synthase silencing. As shown in Figure 3c, *CalS1* mRNA levels were approximately 80% lower in silenced plants compared to control wild-type plants infiltrated with *Agrobacterium* EHA105 alone. These

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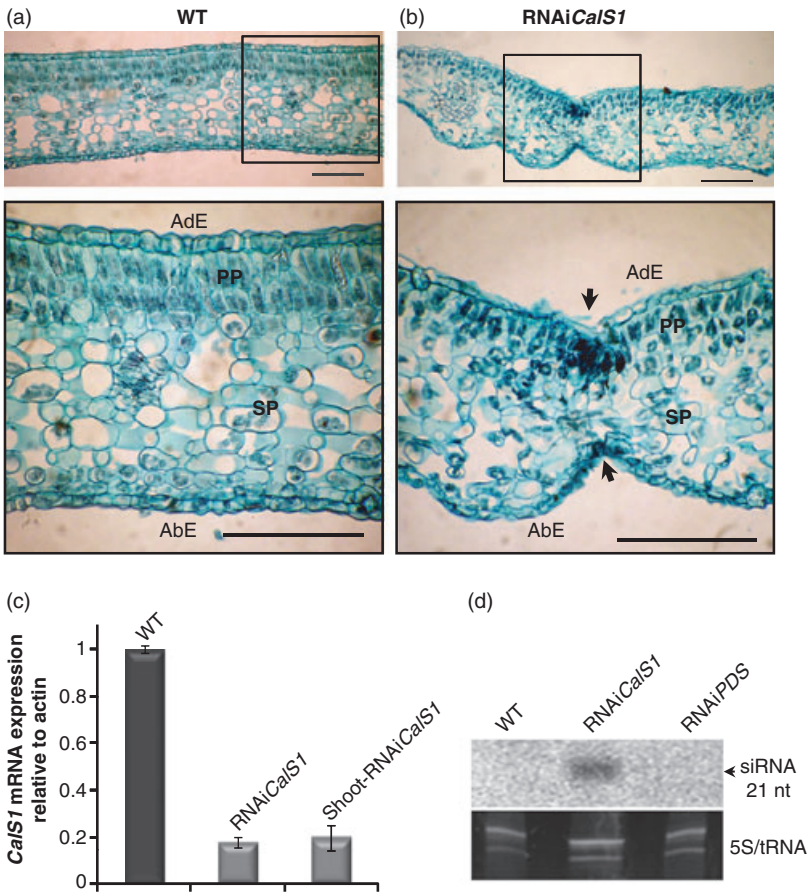

Figure 3 Anatomical structure and *CalS1* RNA levels in lemon leaves silenced with *CalS1*-hpRNAi. (a) A transversal section of a typical lemon wild-type (WT) leaf, magnification 20×. (b) Shortening of palisade cells and tangential flattening of spongy cells in RNAi*CalS1*-silenced leaf, magnification 20×. Insets show the amplification of the section tissue from figures a and b. AdE, adaxial epidermis; AbE, abaxial epidermis; PP, palisade parenchyma; SP, spongy parenchyma. Scale bar 100 μm. Arrows show the notches in the AdE and AbE. (c) Quantitative RT-PCR analysis of the *CalS1* mRNA steady state levels in systemic RNAi*CalS1* leaves. Average cycle threshold values and *n*-fold changes in gene expression in each silencing experiment compared to wild-type (WT) plants agro-infiltrated with bacteria (no vector) were calculated from triplicate samples. Error bars means ± standard deviation of five independent RNAi*CalS1* plants and four independent shoot grafted RNAi*CalS1* plants. (d) Northern blot analysis for the detection of *CalS1* small interfering RNA (siRNA) accumulation levels in wild-type (WT), RNAi*CalS1* and RNAi*PDS* plants at 60 days after agroinfiltration. Total RNA from systemic leaves was isolated and 15 μg was blotted and probed with specific [α-^{32}P] ATP-labelled DNA probes obtained by random priming from the *CalS1* sequences. The size of siRNAs was estimated using labelled 21 nucleotide synthetic RNAs. Ethidium bromide staining of the 5S/tRNA region of the gels is shown below as a loading control. Similar results were obtained with three independent sets of RNA extracts.

variations in *CalS1* transcripts levels correspond with the processing of the double-stranded RNA produced from hpRNA transgenes into 21 nt small interfering RNA (siRNA) in each of the silencing plants (Figure 3d). As expected, no accumulation of *CalS1* 21 nt siRNA was detected either in RNAi*PDS* or wild-type plants (Figure 3d).

Silencing *CalS1* makes citrus plant more susceptible to *Xcc*

We have previously provided evidence that callose is required for resistance to the bacterial pathogen, *Xanthomonas campestris* pv. *campestris*, and that xanthan, the major exopolysaccharide secreted by *Xanthomonas* spp., induces susceptibility to *Xanthomonas campestris* pv. *campestris* in *N. benthamiana* and *Arabidopsis* by suppressing callose deposition (Yun *et al.*, 2006). Recently, we have shown that xanthan-defective mutants of *Xanthomonas axonopodis* pv. *citri* (*Xac* M*gumB,* renamed *Xcc* M*gumB*) are unable to cause disease in *C. limon* and that xanthan plays an important role in the formation of biofilms and in *Xcc* survival prior to development of canker (Rigano *et al.*, 2007), suggesting that callose may be required for resistance to *Xcc* as well.

To investigate this further, we inoculated young leaves of 60-day-old wild-type and RNAi*CalS1* lemon plants with

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bacterial suspensions of M*gumB* and wild-type *Xcc* at a concentration of 10^7 cfu/mL by cotton swab method. A green fluorescent protein (GFP)-labelled strain of *Xcc* and Mgum*B* (Rigano *et al.*, 2007) were used to observe different stages of canker development. Forty-eight hours after infection, the inoculated leaves were stained for the presence of callose with aniline blue and observed by UV-fluorescence microscopy (Figure 4a, d). Callose deposition was visible in wild-type plants inoculated with M*gumB*, as indicated by the bright-light blue stain. No deposition was observed following inoculation with *Xcc* (Figure 4a). Thus, the reduced virulence of M*gumB* associated with the lack of xanthan correlates with callose deposition. Interestingly, RNAi*CalS1* plants showed no callose deposition after infection with M*gumB* (Figure 4d), indicating that the *CalS1* gene is also involved in pathogen recognition in citrus plant. As expected, we also observed no callose deposition when these silenced plants were infected with wild-type *Xcc* (Figure 4d).

At ten to fifteen dpi, leaves of wild-type plants inoculated with *Xcc* showed canker development while M*gumB* showed no symptoms, as has been demonstrated previously (Rigano *et al.*, 2007) (Figure 4b). Interestingly, the canker symptoms in RNAi*CalS1* plants were slightly stronger than in wild-type plants inoculated with *Xcc* (Figure 4b, e). Surprisingly, we observed canker development in *CalS1*-silenced plants inoculated with M*gumB* (Figure 4e). However, at 15 dpi, M*gumB* cankers on RNAi*CalS1* leaves were noticeably different from wild-type *Xcc* cankers. M*gumB* cankers displayed isolated microcolonies of bacteria dispersed within the cankers, suggesting an interruption or nonprogression of canker development (Figure 4e). In comparison, wild-type *Xcc* cankers were fully developed (demonstrated visually by solid fluorescence), and slightly larger in size even compared to the cankers that formed on wild-type plants (Figure 4b, e). Wild-type and RNAi*CalS*1 plants inoculated with 10 mM $MgCl_2$ served as controls and showed no symptoms (data not shown).

To assess the bacterial growth kinetics in RNAi*CalS1* plants and wild-type plants, leaves were inoculated with bacterial suspensions of the *Xcc* and *gumB* mutant as described previously. In wild-type plants, the population size of the *gumB* mutant was significantly lower than *Xcc*, as expected from previous work (Rigano *et al.*, 2007) (Figure 4c). Comparison

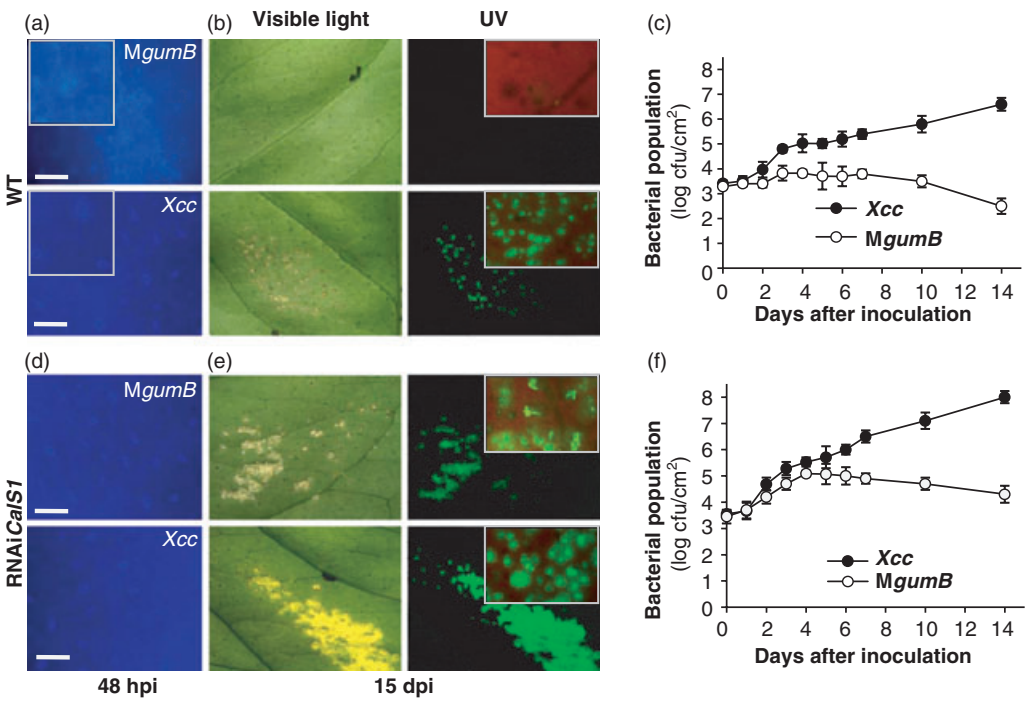

Figure 4 Silencing of *CalS1* in *C. limon* increases susceptibility to *Xanthomonas.* (a, d) M*gum*B and *Xcc* strains were inoculated onto wild-type (WT) and RNAi*CalS1* leaves and were stained with aniline blue at 48- h postinoculation (hpi) to detect callose deposition. Insets show amplification of the callose staining (bright-light blue dots). Scale bars 100 μm. (b, e) Symptom development induced by wild-type strain of *Xcc*-GFP and M*gumB*-GFP strain on lower surfaces of wild-type (WT) and RNAi*CalS1* lemon leaves 15 dpi. Leaves were photographed under white and UV light (520 nm), respectively. GFP-labelled bacteria within the canker pustule are shown enlarged in the top inset. (c, f) Growth *in vivo* of *Xcc* and M*gum*B strains on wild-type (WT) and RNAi*CalS1* leaves, respectively. Values are expressed as means ± standard deviation of three independent experiments.

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of bacterial numbers in RNAi*CalS1* plants, however, revealed no significant differences between the growth of the *Xcc* and *gumB* mutant up to 4–6 dpi. After that time, the population of the *gumB* mutant began to decline and no mutant bacteria could be recovered after 4–5 weeks (data not shown). These results are consistent with the canker symptoms developed by *gumB* mutant on RNAi*CalS1* plants (Figure 4e). Furthermore, the population size of both strains was more than one order of magnitude greater in silenced plants compared to wild-type plants over the 14-day monitoring period (Figure 4f).

CalS1 activity in citrus is insufficient for defence against the xanthan-deficient *Xcc gumB* mutant

We have shown that xanthan produced by *Xcc* suppresses pathogen-associated molecular patterns (PAMPs)-induced signalling in wild-type *C. limon* leaves (Figure 4a, d). To gain better understanding of the defence responses that makes callose-deficient plants more susceptible to *Xcc* infection, we analysed other early host defence markers, including reactive oxygen species levels, measured as hydrogen peroxide. A high accumulation of H_2O_2 in wild-type lemon plants inoculated with the xanthan-deficient *gumB* mutant was observed at 15- h postinoculation, maintaining high levels until 5 dpi (Figure 5a). Interestingly, RNAi*CalS1* leaves inoculated with M*gumB* show a H_2O_2 pattern that is similar to the pattern observed in wild-type plants inoculated with this mutant (Figure 5a). At the beginning of M*gumB* infection, both plants showed higher H_2O_2 accumulation within the guard cells of the stomata, which represent an important entry route for *Xcc*. At 5 dpi with M*gumB*, H_2O_2 production was also observed in the apoplast, which is where *Xcc* wild-type multiplies. The number and intensity of the green fluorescent spots induced by M*gumB* visualized with the H_2O_2-sensitive dye DCFH-2DA was greatly increased both in control wild-type (48.3 ± 6.4%) and RNAi*CalS1* (52.1 ± 5.8%) plants. No H_2O_2 accumulation was observed in wild-type (0.076 ± 0.018%) and RNAi*CalS*1 (0.081 ± 0.015%) leaves inoculated with *Xcc* (Figure 5a). This result confirms that bacterial xanthan production suppresses *Xcc* elicitor-triggered immunity in citrus plants.

To clarify the relationship between accumulation of H_2O_2 and bacterial epiphytic fitness, we analysed the population size of *Xcc* and M*gumB* on the surface of wild-type and RNAi*CalS1* lemon leaves at 5 days postinoculation. As shown in Figure 5b, the population size of *gumB* mutant was much smaller compared to the *Xcc* population

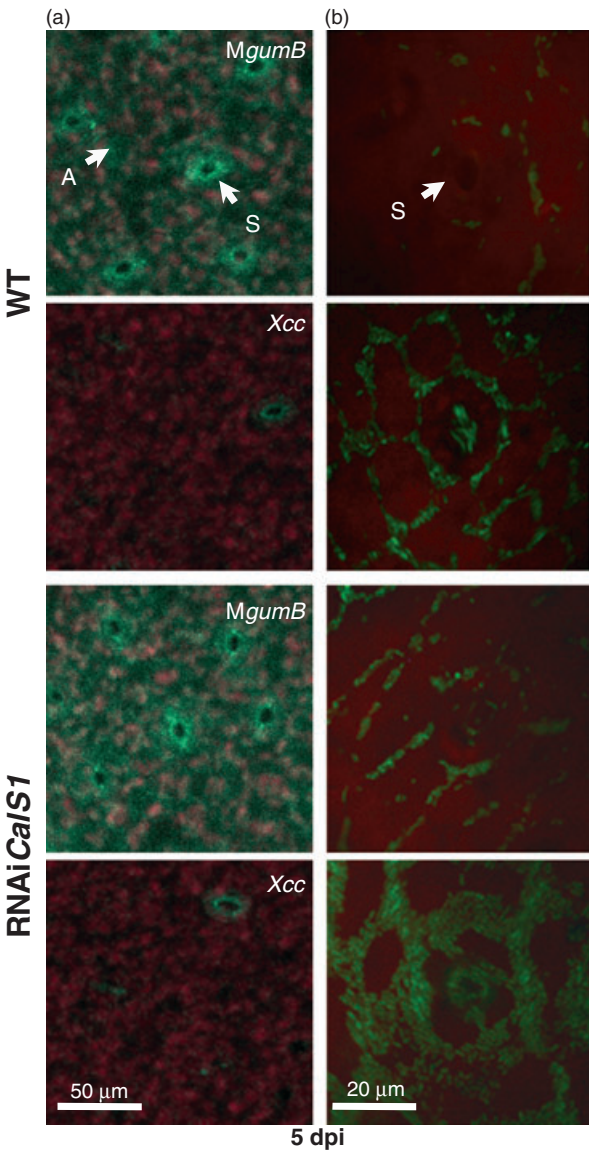

Figure 5 The accumulation of H_2O_2 triggered by the xanthan-deficient mutant (M*gumB*) does not required *CalS1* expression. (a) M*gum*B and *Xcc* strains were inoculated onto wild-type (WT) and RNAi*CalS1* leaves and stained with 2′,7′-dichlorofluorescein diacetate to detect H_2O_2 accumulation at 5 days postinoculation (dpi). Leaves were photographed using confocal laser scanning microscopy. Red indicates chlorophyll autofluorescence. Magnification 50×; Scale bars, 50 µm. (b) Epiphytic population of *Xcc* and the M*gumB* mutant on superficial lemon leaf at 5 dpi. GFP-labelled bacteria were detected by fluorescence microscopy. Magnification 100×; Scale bars, 20 µm. Four independent biological assays were carried out for each strain using five wild-type and five RNAi*CalS1* plants (three leaves per plant) with essentially the same outcome. Stomata (S) and apoplast (A) are indicated by arrows.

size across both wild-type and RNAi*CalS1* leaves, and both leaf types displayed higher H_2O_2 levels following inoculation with the M*gumB* mutant compared to inoculation

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with *Xcc*. Irrespective of bacterial strain, more aggregates were seen on RNAi*CalS*1 leaves than on wild-type leaves at early stages postinfection. These results are consistent with the results shown in Figure 4 and suggest that callose deposition is a rapid and specific cell wall response during *Xcc* infection.

Taken together, these findings indicate a greater susceptibility of RNAi*CalS*1 plants compared with wild-type plants to *Xcc,* confirming a role for CalS1 in callose synthesis during citrus defence against this pathogen. However, these findings also suggest that without bacterial xanthan production, inhibition of callose deposition is not enough to allow bacterial growth, as evidenced by the limited growth of the M*gumB* mutant on RNAi*CalS*1 leaves. A likely explanation is that in the absence of callose depostion, other defence signalling molecules or signalling triggered by H_2O_2 itself are eventually able to contain the pathogen (Quan *et al.*, 2008). This does not occur with the wild-type *Xcc* strain because xanthan successfully suppresses the defence response.

Discussion

An optimized method for RNAi induction in citrus via hpRNA expression

Gene silencing by transient expression of RNAi-inducing hairpin RNA does not require stable genetic transformation and is consequently a very promising technique for functional genomic analysis in citrus. In this work, we optimized the hpRNA silencing system (Wesley *et al.*, 2001; Helliwell and Waterhouse, 2003) in *C. limon* and other members of the *Rutaceae* family to allow for functional analysis of candidate genes. We first evaluated the silencing system using lemon endogenous *PDS*, a gene that has been used for this purpose in many plant species (Kumagai *et al.*, 1995; Ruiz *et al.*, 1998; Liu *et al.*, 2002; Brigneti *et al.*, 2004). Upon induction of *PDS* silencing with these constructs, we observed different grades of photobleaching. Plants most severely afflicted died following complete photobleaching. In milder cases, silencing in the upper leaves did not persist and only leaves from one new lateral branch displayed photobleaching after pruning (Figure 1). Approximately 80% of silenced plants showed a strong photobleaching phenotype in systemic leaves that persisted for at least a year postinoculation (Figure 1). The demonstrated success of *PDS* systemic silencing in citrus provides a basis for analysis of citrus gene function, metabolic routes and signalling pathways using RNAi.

Role of callose in citrus development

We exploited the high-throughput capability of the hairpin RNA vector and the availability of partial *C. reshni CalS* cDNA library to test possible roles for this gene in cell wall development and defence responses to *Xcc* in lemon plants.

RNAi*CalS1 C. limon* plants were generated by transient expression of hpRNA from the pHG12 *Cl-CalS1* vector, which contains the endogenous *C. limon CalS1* target gene sequence (Figure 2). In these systemic silencing leaves, expression of the construct reduced *CalS1* mRNA levels to 20% tested by qRT-PCR. This can be explained by the accumulation of *CalS1* 21 nt siRNAs (Figure 3) (Hamilton and Baulcombe, 1999; Hamilton *et al.*, 2002). As shown in Figures 2 and 3, RNAi*CalS* silencing citrus plants led to alterations at both gross phenotypical and histological levels. Silenced plants were shorter than control plants and their leaves were wrinkled and smaller. A similar phenotype was reported in cellulose synthase-silenced *N. benthamiana* (Burton *et al.*, 2000). Recently, it was reported that *CalS1* gene expression is regulated in a tissue-specific manner in both the leaves and root hairs of *Arabidopsis* (Dong *et al.*, 2008). Histological analysis of RNAi*CalS1 C. limon* leaves showed morphological alterations both in shape and organization of cells. It was suggested that the formation of a functional CalS1 complex is vital to building a cell plate, which must be completed in a short time, and that imbalanced synthesis of callose may alter the composition of the cell plate producing daughter cells with altered cells walls (Verma and Hong, 2001). We hypothesize that these alterations are caused by a temporary loss of cell wall strength or rigidity enabling abnormal cell expansion in lemon leaves.

CalS1 is required to defend against *Xcc* infection in citrus plants

The greater susceptibility of RNAi*CalS1* plants to *Xcc* compared to wild-type plants suggests that CalS1 plays a role in citrus defence against this pathogen (Figure 4). Recently, Lee *et al.* (2009) studied the ultrastructural aspects of citrus canker development in unwounded leaves of citrus species. They showed that in leaves from the susceptible species of Mexican lime, *Xcc* invasion and extracellular multiplication were usually accompanied by host cell wall dissolution and a disruption of the host cell. Local accumulation of papillae-like materials within the host cells and in close proximity to bacteria was observed in the

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mesophyll tissues of more resistant species, such as Yuzu (*C. ichangensis* X *C. reticulata* var. *austere*). Based on our findings and the results obtained by Lee *et al.* (2009), we propose that callose contributes to penetration resistance against invading bacterial mesophyllic pathogens by providing a physical barrier. In callose-silenced citrus plants, the consequent reduction in callose levels may weaken this barrier leading to enhanced *Xcc* susceptibility compared to wild-type plants.

Recently, Dong *et al.* (2008) reported a significant induction of *CalS1* and *CalS12* genes in *Arabidopsis* by salicylic acid treatment and upon *Hyaloperonospora arabidopsis* infection. Interestingly, the *cals12 Arabidopsis* mutant not only failed to synthesize callose at papillae but was also more resistant to the fungus compared to the *cals1 Arabidopsis* mutant and wild-type plants (Dong *et al.*, 2008). These surprising results are consistent with the two other recent reports on *Arabidopsis pmr4/gsl5* (*cals12*) mutants (Jacobs *et al.*, 2003; Nishimura *et al.*, 2003), in which these plants were found to be more resistant to fungi powdery mildew and downy mildew. In contrast, Vogel and Somerville (2000) observed greater susceptibility in *Arapidopsis* using the fungus *Erysiphe cichoracearum*. To explain these seemingly contradictory findings, it has been proposed that pathogen-induced callose may negatively regulate the salicylic acid signalling pathway of the plant and that the lack of callose in these mutants may therefore suppress pathogen infection (Nishimura *et al.*, 2003; Dong *et al.*, 2008).

Taken together, these results suggest that different CalS isoforms may be required for callose production in specific tissues and that callose may play multiple roles depending on the pathogen and host.

Death of M*gumB* mutant is because of oxidative burst rather than callose deposition

Xanthan plays an important role in biofilm formation and canker development on lemon leaves (Rigano *et al.*, 2007). Here, we have shown that the reduced virulence of M*gumB*, associated with the lack of xanthan, correlates not only with callose deposition but also with high generation of H_2O_2 in wild-type plants (Figures 4 and 5). These results suggest that another role of xanthan in bacterial canker disease is to suppress defence induction in citrus, which supports earlier findings on the role of callose in plant defence to bacterial pathogens (Yun *et al.*, 2006; Aslam *et al.*, 2008).

Surprisingly, the canker symptoms induced by the *gumB* mutant on RNAi*CalS1* lemon leaves at 15 dpi were not fully developed (Figure 4). This correlates with the low cell number observed on the leaf surface of these plants at an earlier time postinfection (Figure 5b). Moreover, plants inoculated with the xanthan-deficient mutant failed to induce callose deposition, but showed no alteration in H_2O_2 production (Figures 4 and 5).

Together, these results indicate that M*gumB* can colonize RNAi*CalS1* tissue, but that without xanthan the bacteria are unable to survive for a long period (e.g. beyond 30–35 days) and that defence triggering induced by PAMPs kills the bacteria. The different outcome of the M*gumB* mutant compared to wild-type *Xcc* may reflect the multiple roles of xanthan in plant disease. Possible roles may include contribution to bacterial survival within leaves, for example through biofilm formation, the ability to retain water and resistance to abiotic stress (Rigano *et al.*, 2007), as well as suppression of additional plant defence responses, such as callose deposition and H_2O_2 accumulation.

In conclusion, we have developed an efficient method to study gene function in *C. limon* using agroinfiltration to transiently silence endogenous genes. The silencing approach should allow further study of the function of genes in citrus beyond the *CalS* gene family. Currently, an abundance of citrus ESTs are available in a public database, covering an array of citrus varieties and responses to abiotic and biotic stresses (International Citrus Genome/Genomics Consortium, http://www.citrusgenome.ucr.edu/). This sequence availability, combined with the successful hpRNA vector in *C. limon* demonstrated here, should facilitate the study of gene function in citrus interactions not only with *Xcc* but also with a range of other pathogens. Moreover, knocking out the expression of host genes with a role in canker disease will likely be an important tool in development of new and sustainable strategies to manage the disease.

Experimental procedures

Plant material, pathogens and pathogenicity assays

Plantlets from seed germination of *Citrus lemon* (L.) Burm f. (cv. Eureka Frost and cv. Lisboa Frost) and Troyer citrange (*Poncirus trifoliata* (L.) Raf. x *Citrus sinensis* (L.) Osb.) were used for silencing assays. Seeds were collected in Tucumán and provided by the Estación Experimental Agroindustrial Obispo Colombres, Tucumán, Argentina. After disinfection with 0.5% v/v of sodium hypochlorite for 15 min, seeds were germinated in a 1 : 1 mixture

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of compost and peat. The plantlets were grown as previously reported (Siciliano *et al.*, 2006). Propagation of silencing was evaluated by grafting buds of silenced lemon plantlets onto non-silenced Troyer citrange rootstocks using the grafting standard procedure. Average grafting success was 30–50% from 30 grafts. Grafted plants were acclimatized in a greenhouse and were tested 3–6 months after the bud break for the silenced phenotype.

The GFP-labelled bacteria of *Xanthomonas citri* subsp. *citri* (*Xcc*) wild-type strain and the mutant strain defective in production of the extracellular polysaccharide xanthan, *Xcc gumB* (M*gum*B), were previously described (Rigano *et al.*, 2007). Bacterial suspensions of *Xcc* and *gumB* mutants (10^7 cfu/mL in 10 mM $MgCl_2$) were inoculated onto the surface of lemon leaves by cotton swab. Inoculated plants were maintained for 25–30 days in a growth cabinet, with temperatures ranging from 25 to 28 °C, high humidity, a photoperiod of 16- h light and a light intensity of 150–200 μE/s/m^2. Disease progression was monitored phenotypically in three separate biological assays and through analysis of bacterial growth curves (Rigano *et al.*, 2007).

Construction of hairpin RNA expression vectors

The pHELLSGATE12 vector (CSIRO Plant Industry, Camberra, Australia) (Helliwell and Waterhouse, 2003) and the Gateway recombination system from Invitrogen (http://www.invitrogen.com/gateway) were used to generate the RNAi constructs, whereby a PCR product amplified from the target gene is first inserted into the donor pENTR3C vector (Invitrogen, Carlsbad, CA, USA) between *att*L1/*att*L2 sites. The gene fragment from the intermediate clone is then inserted into the pHELLSGATE12 vector by recombination between *att*L1/*att*L2 and attR1/attR2, mediated by LR Clonase (Invitrogen). When the construct is expressed in plants, a hairpin RNA (hpRNA) with the intron spliced out is produced.

Fragments of 386 bp from the *Citrus limon* phytoene desaturase (*Cl-PDS*) coding region were PCR amplified using the primer pairs, 5′-GCCATGTCAAAGGCACTAAA-3′ (PDS-R) and 5′-ATGACTGGAACTCCCACCAA-3′ (PDS-F), designed from *PDS* ESTs sequences of *C. sinensis* (DQ235261). The fragments of 398 bp from the *C. limon* callose synthase (*Cl-CalS*) coding region were PCR amplified using the primer pairs, 5′-CCGCCCATAAAGAAACACAT-3′ (CalS-R) and 5′-CCGCCCATAAAGAAACACAT-3′ (CalS-F), designed from *CalS* ESTs sequences of *C. reshni* (CX305437). The *Cl-PDS* and *Cl-CalS* PCR products were purified and cloned into the pGEM-T Easy vector (Promega, Mannheim, Germany) to produce pGEM *Cl-PDS* and pGEM *Cl-CalS*, respectively. These constructs were then sequenced, digested with *Eco*RI and sub-cloned into the cleavage site, *Eco*RI, of the pENTR3C vector (Invitrogen). Recombination reactions from pENTR *Cl-PDS* and pENTR *Cl-CalS* clones with pHELLSGATE12 vector were carried out according to Helliwell and Waterhouse (2003). Transformed *Escherichia coli* DH5α cells were selected on LB media (Sambrook *et al.*, 1989) supplemented with 100 μg/mL spectinomycin. The presence and orientation of the insert was confirmed by restriction digest with *Xho*I and *Xba*I of the plasmid DNA (Helliwell and Waterhouse, 2003). Between 70% and 80% of the clones obtained contained the pyruvate dehydrogenase kinase (PDK) intron in reverse orientation (pHG12 C*I-TARGET GENE*-R) with respect to the promoter. The remaining 20% contained the intron in forward orientation (pHG12 C*I-TARGET GENE*-F).

Agroinfiltration-mediated delivery of dsRNAs

Agrobacterium tumefaciens EHA105 cells (Hood *et al.*, 1993) transformed by electroporation (Dower *et al.*, 1988) with either the pHG12 *Cl-PDS* construct or the pHG12 *Cl-CalS1* construct were inoculated into 10 mL of LB medium (Sambrook *et al.*, 1989) supplemented with 25 μg/mL nalidixic acid and 100 μg/mL spectinomycin and grown at 28 °C to saturation. Cells were precipitated and resuspended to a final concentration of 0.8 OD_{600} in a solution containing 10 mM $MgCl_2$ and 150 μM acetosyringone (Sigma-Aldrich, St Louis, MO). The cultures were incubated at room temperature for 6 h before agroinfiltration. The agroinfiltration into *C. limon* plants at the four- to six-leaf stage was performed as described previously (Siciliano *et al.*, 2006). Each construct was infiltrated into five plants grown from seeds.

Real-time quantitative reverse transcription–PCR

Total RNA from control and silenced lemon leaves was isolated according to the manufacturer's instructions (RNeasy Plant Mini Kit; Qiagen, Valencia, CA), following treatment with RNase-free DNase (Promega, Mannheim, Germany). Reverse transcription was performed by M-MLV reverse transcriptase (Invitrogen) with 1 μg DNase-treated total RNA and oligo-dT12-18 as primers. Synthesized cDNA was used for quantitative reverse transcription-polymerase chain reaction (qRT-PCR). The reactions were carried out in the presence of the double-stranded DNA-specific dye SYBR green (Invitrogen) and monitored in real time with the Mastercycler ep *realplex* real-time PCR system (Eppendorf, Hamburg, Germany). The following oligonucleotides were used in this study: *PDS*: PDS-F and PDS-R; *CalS*: CalS-F and CalS-R. A lemon actin amplicon used as internal standard for quantifications was amplified using 5′-TTTACCACCACAGCCGAACG-3′ (ACT-R) and 5′-TGGAGCCACGACCTTGAT-3′ (ACT-F). All of these amplicons have a product size approximately of 400 bp. PCRs were performed for 40 cycles according to the following conditions: denaturation at 95 °C for 15 s, annealing at 55 °C for 30 s and extension at 72 °C for 40 s. After amplification, melting-curve analyses were performed to exclude artefactual amplifications. The relative expression of transcripts RNA was calculated using the threshold cycle values (Ct) obtained from each sample as follows: relative expression = $2^{-\Delta\Delta}$, being $\Delta Ct = Ct_{\ sample} - Ct_{\ actin}$ and $\Delta\Delta Ct = \Delta Ct_{\ sample} - \Delta Ct_{\ ref\ sample}$. Nonsilenced lemon leaves served as the reference sample, and the results were normalized against actin. The average values were calculated from triplicate samples.

Small RNA Northern blot

Total RNA from *Citrus limon* leaves was isolated using TRIzol reagent (Invitrogen) followed by separation on 17.5% denaturing polyacrylamide gels. RNAs were transferred to Hybond N+ membrane (Amersham Pharmacia Biotech, Bucks, UK) in TBE 0.5× for 1 h using the Bio-Rad semi-dry transfer unit at 300 mA at 25 °C. Membranes were fixed by UV cross-linking at 1200 μJoules × 100 (UVP UV Crosslinker, UK). The blots were hybridized with [α-^{32}P] ATP-labelled DNA probes of *Cl-CalS* generated by random priming

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from the respective cDNA clones. Both prehybridization and hybridization reactions were performed as described in Hamilton and Baulcombe (1999) at 40 °C. Radioactivity in bands was detected using a phosphorimager Storm 840 (Amersham Pharmacia Biotech, Bucks, UK).

Histological and anatomical assays

Citrus limon plants agro-infiltrated with either pHG12 *Cl-CalS1* or *Agrobaterium* EHA105 alone were photographed at 50 days post-inoculation. The internode length was measured with the ImageJ software v1.41 (National Institutes of Health, Bethesda, MD, USA). Data were analysed with analysis of variance (one-way ANOVA). This analysis was carried out with five RNAi*CalS1* plants and five control wild-type plants in three replicate experiments.

Histological staining employed young leaves (50%–80% expansion) that were cut from 60-day-old *CalS1*-silenced and wild-type plants. All materials were immediately immersed in a fixative containing 10% formaldehyde, 5% acetic acid and 50% ethanol. After the material was dehydrated through a graded ethanol series, the leaves were passed through graded solutions of xylene in absolute ethanol and immersed in several changes of melted paraffin in xylene (65 °C) (Berlyn and Miksche, 1976). The paraffin-embedded samples were traced on 10- μm-thick paraffin sections, using a Minot 1212 microtome (Leitz, Wetzlar, Germany). The tissues were stained with zafranin and fast-green and mounted in Canada balsam (Strittmatter, 1979).

H_2O_2 and callose detection

Histochemical detection of H_2O_2 was carried out as described by Sanchez *et al.* (2010) after staining with fluorescence marker 2′,7′-dichlorofluorescein diacetate (DCFH-DA; Sigma-Aldrich) (Ezaki *et al.*, 2000). H_2O_2 accumulation was quantified by comparing the number of green fluorescent pixels following M*gumB* inoculation to the number of green fluorescent pixels following *Xcc* inoculation relative to the total pixel count on digital photographs of treated leaf areas using the program ImageJ software v1.41 (National Institute of Health). The experiment was repeated on at least five different plants, and three leaves per plant were examined and photographed under UV light with an inverted confocal microscope (Nikon C1, Japan). The images were generated by using Nikon EZ-C1 Version 3.90. Aniline blue staining for the presence of callose deposition in *C. limon* leaves was performed as described previously (Yun *et al.*, 2006). Leaves were examined and photographed under UV light with an epifluorescence microscope (BH2; Olympus Optical Ltd. Company, Tokyo, Japan).

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