Research Article

Type IV Secretion System Is Not Involved in Infection Process in Citrus

Tiago Rinaldi Jacob,1 Marcelo Luiz de Laia,2 Leandro Marcio Moreira,3 Janaína Fernandes Gonçalves,2 Flavia Maria de Souza Carvalho,4 Maria Inês Tiraboschi Ferro,1 and Jesus Aparecido Ferro1

1 Faculdade de Ciências Agrárias e Veterinárias de Jaboticabal, Departamento de Tecnologia, Universidade Estadual Paulista (UNESP), 14.884-900 Jaboticabal, SP, Brazil
2 Departamento de Engenharia Florestal, Faculdade de Ciências Agrárias, Universidade Federal dos Vales do Jequitinhonha e Mucuri, 39.100-000 Diamantina, MG, Brazil
3 Departamento de Ciências Biológicas (DECBI), Instituto de Ciências Exatas e Biológicas (ICEB) and Núcleo de Pesquisas em Ciências Biológicas (NUPEB), Universidade Federal de Ouro Preto, 35.400-000 Ouro Preto, MG, Brazil
4 Embrapa Informática Agropecuária, CNPTIA-EMBRAPA, Campinas, SP, Brazil

Correspondence should be addressed to Jesus Aparecido Ferro; jesus@fcav.unesp.br

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The type IV secretion system (T4SS) is used by Gram-negative bacteria to translocate protein and DNA substrates across the cell envelope and into target cells. Xanthomonas citri subsp. citri contains two copies of the T4SS, one in the chromosome and the other is plasmid-encoded. To understand the conditions that induce expression of the T4SS in Xcc, we analyzed, in vitro and in planta, the expression of 18 ORFs from the T4SS and 7 hypothetical flanking genes by RT-qPCR. As a positive control, we also evaluated the expression of 29 ORFs from the type III secretion system (T3SS), since these genes are known to be expressed during plant infection condition, but not necessarily in standard culture medium. From the 29 T3SS genes analyzed by qPCR, only hrpA was downregulated at 72h after inoculation. All genes associated with the T4SS were downregulated on Citrus leaves 72h after inoculation. Our results showed that unlike the T3SS, the T4SS is not induced during the infection process.

1. Introduction

Xanthomonas citri subsp. citri (Xcc) is a Gram-negative plant pathogenic bacteria that causes severe disease in many economically important citrus plants [1]. The citrus canker induced by Xcc is a destructive disease characterized by canker lesions on leaves, stems, and fruits; furthermore the pathogen induces defoliation, which results in reduced yield and premature fruit drop [2]. Control is difficult in areas where the disease is already established, and plant eradication is the only effective way to control and prevent the disease spread. Recurrent and severe attacks of the pathogen are responsible for serious economic losses in citrus groves around the world [2]. The focus of the present study is aimed at understanding the role of bacterial secretory systems, specifically the type IV secretory system (T4SS). Seven secretory systems are known and described in prokaryotic organisms, and each one is related to a physiological process [3–5]. Of these, the best studied is the type III secretory system (T3SS), which enables bacterial pathogens to deliver effector proteins into eukaryotic cells [6]. Some bacterial pathogens, including species from Chlamydia, Xanthomonas, Pseudomonas, Ralstonia, Shigella, Salmonella, Escherichia, and Yersinia, depend on the T3SS to induce damage to the host. In contrast, other organisms including Agrobacterium tumefaciens, Helicobacter pylori, and Legionella pneumophila depend on the type IV secretory system (T4SS) for virulence induction [7].
The T3SS contains at least twenty distinct proteins, which are subdivided into three parts. The basal body of the system [8–11] is associated with an ATPase and likely facilitates the entry of substrates into the secretion apparatus [12]. This basal body is also associated with a protein needle (the second part of the T3SS), which binds the bacterium to host cells and acts as a conduit for effectors secretion. The third part of the T3SS is composed of three proteins that are exported through the bacterial needle and form a pore on the surface of the host cell, which facilitates the export of toxins into the target cytoplasm [13].

On the other hand, the T4SS is a multiprotein complex that consists of a protein channel (encoded by virB and virD) through which proteins or protein-DNA complexes can be translocated between bacteria (cell-to-cell communication) and into host cells [14]. Translocation is driven by a number of cytoplasmic ATPases that potentially energize large conformational changes in the translocation complex [15]. There are three functional types of T4SS. The first type, found in many Gram-positive and Gram-negative bacteria and some Archaea, functions in conjugation and in the transfer of T-DNA into plant cells by A. tumefaciens. A second type of T4SS mediates DNA uptake in the transformation process (found in H. pylori). A third type of T4SS is used to transfer toxic effector proteins or protein complexes into the cytoplasm of host cells (found in Bordetella pertussis, Legionella pneumophila, Pseudomonas aeruginosa, and Xcc) [15].

Substrates transported by the T4SS modulate various cellular processes including apoptosis, vesicular traffic, and ubiquitination; furthermore, the number of type IV effector proteins continues to increase [16–19]. However, most of these substrates have not been functionally characterized, and their role in bacterial pathogenesis remains unknown [20].

In many bacteria, these two secretory systems types are well characterized; however, in Xanthomonas spp. only the T3SS has been intensively studied [1], in contrast to the limited number of studies involving the T4SS [21, 22]. In Xcc the T4SS deserves more attention, especially since the genome contains two copies of this system (chromosomal and plasmid-borne) [23]. The plasmid copy is homologous to others Xanthomonads that contain the T4SS on extrachromosomal DNA. However, only Xcc and Xanthomonas campestris contain the chromosomal copy of the T4SS.

To understand the conditions that stimulate expression of T4SS genes in Xcc we used qPCR to analyze the in vitro and in planta expression of 18 ORFs from the T4SS (both chromosomal and plasmid copies) and 7 hypothetical ORFs flanking the T4SS. To validate the qPCR data, we compared expression of 29 ORFs from the T3SS, which are known to be expressed in planta.

Our results showed that the T4SS is not induced during the infection process in Xcc, but may be very important in cell-to-cell communication.

2. Materials and Methods

2.1. Microbiological Procedures. Xcc strain 306 was previously sequenced by da Silva et al. [23]. Xcc was maintained in sterile tap water and grown on nutrient agar (NA) medium containing 3 g beef extract, 5 g peptone, and 15 g agar in 1 L of distilled water at 28°C. After 24 h, three single colonies were transferred to new NA plates and incubated for 12 h at 28°C. These Xcc solid cultures were used as pre-inoculum for in planta and in vitro studies. For in planta studies, pre-inoculum cultures were aseptically transferred to sterile flasks containing 50 mL of nutrient broth (NB) and the bacterial concentration was adjusted to 10⁶ CFU/mL (OD₆₀₀ = 0.3). This bacterial suspension was taken up in a 1 mL needleless syringe and used to infiltrate orange leaves (Citrus sinensis cv. pera) grown in 20 L capacity pots. A total of nine plants were inoculated (20 leaves per plant) and maintained for 72 h in a growth room at 28°C, with a 12 h photoperiod and light intensity ~2,000 lux. Three plants were used for each incubation period. After multiplication, inoculated leaves were collected, sliced into thin strips with a razor blade, and placed in a beakers with sterile distilled water in an ice bath with gentle agitation. Samples from each plant were placed in separate beakers for bacterial exudation. After 5 min, leaf debris was removed by filtration through gauze, and bacterial cells were recovered by centrifugation at 5,000 × g for 5 min at 4°C. Total RNA was extracted immediately, as described below. For in vitro studies, 1 mL of each Xcc suspension at 10⁶ CFU/mL were aseptically transferred to three Erlenmeyer flasks and incubated for 12 h at 28°C with shaking (200 rpm). After incubation, bacterial cells were harvested by centrifugation at 5,000 × g for 5 min. Total RNA was extracted as described below. Thus, we obtained three independent biological replicates for Xcc growth in vitro and in planta. All cultivation media were obtained from Difco Chemical Co., Detroit, USA. For confirmatory RT-qPCR, the same procedures were followed except the plant incubation period was only 72 h, due to RNA quality and concentration sufficient to yield reliable results. The time point 72 hours was the shortest period to allow obtaining satisfactory bacterial mass to the proposed analyzes involving gene expression. The bacterial cells concentration in short time (12 or 24 hours) don’t allows this study. For this reason, some works are using culture media that mimic vegetable conditions to investigate the infectious process under these time scales [24, 25].

2.2. Extraction of RNA from Xcc. Each inoculated flask represented one independent biological replicate. RNA was extracted using an Illustra-RNAspin Mini RNA Isolation Kit (Amersham Biosciences) following the manufacturer’s instructions. To ensure that samples did not contain DNA, PCR was performed using RNA samples treated with DNase I as a template. PCR conditions consisted of an initial denaturing step of 94°C for 3 min, followed by 35 cycles of a denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and elongation at 72°C for 2 min. A final elongation step of 72°C for 4 min was performed, and then samples were maintained at 4°C until needed. The amplification reaction was conducted in a total volume of 25 μL containing 200 ng RNA in 2.5 μL, PCR buffer (2.5 μL, Invitrogen), 1.5 mM MgCl₂, 0.2 mM dNTP, 300 nM 16S rRNA primer [26], and 1 U Taq DNA polymerase (Invitrogen). The products were electrophoresed in a 1% agarose gel with TAE buffer, stained
with ethidium bromide, and visualized using a UV transilluminator. No products were observed (data not shown). To verify the quality of extracted RNA, the samples were analyzed by electrophoresis in a 1% agarose gel using TAE buffer followed by ethidium bromide staining. The A260/280 ratio of RNA samples was measured, and RNA was quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). RNA samples were stored at −80°C until needed.

2.3. Gene Expression Using RT-qPCR. First strand cDNA synthesis and all RT-qPCR reactions were done using the SuperScript III First-Strand Synthesis SuperMix for RT- qPCR (Invitrogen) as recommended by the manufacturer’s specifications except for the amount of cDNA in each reaction, which was 20 ng. All PCR was performed with SYBR Green on a 7500 Real-Time PCR instrument (Applied Biosystems) using three biological replicates and three technical replicates (one for each biological replicate). PCR was for 2 min at 50°C, 10 min at 95°C, followed by 40 cycles of 15 s at 95°C, and 1 min at 60°C. To determine PCR efficiency, standard curves were generated using cDNA samples at five dilutions and measured in triplicate. rpoB, atpD, and gyrB were used as reference genes in all experiments [26]. To perform relative expression analysis, we used the 2^−ΔΔCT method [27]. Primer features are presented in Table 1.

3. Results and Discussion

3.1. qPCR Results for the T3SS. The results obtained from Xcc growing in planta or in vitro are presented in Figure 1. From the 29 T3SS genes analyzed by qPCR, only hrpA was downregulated at 72 h after inoculation (Figure 1(b)). hpaA, hpaL, hpaB, hpaD6, hrpE, and hrpF showed the highest rates of expression, whereas phaE, hrpG, hrpX, and hrcA showed the lowest levels of expression. Interestingly, hrpB4 and hrpXct, which were shown to be necessary for Xcc pathogenesis [28], were not among the most upregulated genes in this study.

3.2. qPCR Results for the T4SS. The results for the expression levels of T4SS genes in planta and in vitro are shown in Figure 2. All genes associated with the T4SS were downregulated on Citrus leaves 72 h after inoculation (Figure 2(c)). We also analyzed the expression of hypothetical genes located near virB in chromosomal DNA (XAC2606, XAC2611, XAC2613, and XAC2622) and in plasmid pXAC64 (XACb0035, XACb0042, XACb0043, XACb0048, and XACb0049) (Figure 2(d) and Table 1). ORFs representing hypothetical genes in both the chromosome and plasmid were down-regulated when Xcc was cultivated in Citrus sinensis for 72 h. An exception was XAC2611, where expression was similar when Xcc was cultivated in culture medium or in planta (Figure 2(d)).

Our results clearly show different gene expression profiles for the T3SS and T4SS in Xcc during Citrus infection. The T4SS was previously described in A. tumefaciens, where it mediates the transfer of DNA and protein substrates to plants and other organisms via a cell contact-dependent mechanism [14]. In Xcc, the genome sequence revealed the presence of two virB operons, one on the chromosome and a second copy in the 64 kb plasmid pXAC64 [23]. The chromosomal genes virB1, virB3, virB4, virB8, and virB9, and the plasmid-encoded genes virB1, virB2, virB4, virB6, virB9, and virB11 were all down-regulated in planta (Figure 2(c)). This was somewhat surprising because the T4SS is essential for a successful infection in many Gram-negative pathogenic bacteria [29]. The fact that ORFs representing hypothetical genes in both the chromosome and plasmid near the virB genes were also down-regulated under the same condition (Figure 2(d)) indicates that they could be related to the T4SS system in Xcc. Recent studies in Brucella suis [30] and in A. tumefaciens [31] showed that alterations in VirB8 result in protein dimerization, a process that modifies the T4SS structure and affects bacterial virulence. In Streptococcus suis [32], the knockout of the virD4-89K and virB4-89K of the T4SS eliminated the lethality of a highly virulent strain and impaired its ability to trigger host immune responses in mice. Recent studies continue to demonstrate the importance of the T4SS and its components in virulence; thus the lack of induction of Xcc T4SS genes in the present study is intriguing. However, in Xanthomonas campestris pv. campestris T4SS-deletion mutant displayed the same virulence as wild type and authors conclude that T4SS is not involved in pathogenicity in that Xanthomonas [33].

Wang et al. [34] presented evidence that in planta transfer of a 37 kb plasmid (pXcB) from Xanthomonas aurantifolii to Xanthomonas citri can occur via T4SS. Thus, at least the T4SS copy present in Xcc plasmid can play a role in horizontal gene transfer. All but one of the twenty-nine genes from the T3SS of Xcc were up-regulated in planta. Laia et al. [28] showed that Xcc mutants containing mutations in hrpB4 or hrpXct failed to cause disease and growth in citrus leaves was lower than the wild-type Xcc strain 306. These two genes, which are not among the most up-regulated genes in the present study, are part of the hrp (hypersensitive reaction and pathogenicity) system and comprise part of the T3SS [35]. In the related pathogen, Xanthomonas campestris pv. vesicatoria (Xcv), a hrpB4 mutant was unable to cause disease in susceptible pepper plants or the hypersensitive reaction in pepper plants carrying the respective compatible R gene [36]. Previously, hrpXcv was shown to be necessary for transcriptional activation of five hrp genes (loci hrpB to hrpF) [37], and hrpB4 was required for complete functionality of the T3SS in Xcv [36]. Thus it is apparent that a gene does not have to be strongly up-regulated during infection to play an important role in virulence. Multiple genes, including hpaA, hpaE, hpaF, hpaP, hpaB, hrpB1, hrpB2, hrpB7, hrpD5, hrpM, hrpW, hrcC, hrcQ, hrcS, hrcT, and hrcV, were expressed at levels similar to hrpXct and hrpB4 and are good candidates for further studies into their roles in citrus canker disease.

Among the most up-regulated genes, hrpD6 warrants further attention. A hrpD6 mutant of Xanthomonas oryzae pv. oryzae (Xoo) failed to trigger a hypersensitive response in tobacco and was nonpathogenic in rice because the mutation in hrpD6 impacts the secretion of T3SS effectors, such as HpaL, which is translocated through the T3SS [38].
| XAC ID | Gene  | Gene ID | Product (NCBI Annotation) | Primers (Forward) | Primers (Reverse) | Amplicon (bp) | System |
|--------|-------|---------|--------------------------|-------------------|-------------------|--------------|--------|
| XAC0293 | hrpB  | 1154364 | ATP-dependent RNA helicase | GCCGTATCCCGTTGACCTT | ACCGCCGCCCTCATCTCTTTT | 81           | T3SS   |
| XAC0393 | hpaF  | 1154464 | HpaF protein             | GCGGAGCGTTTGGACAA  | CCGGAGATCGCGGCAATT | 57           | T3SS   |
| XAC0394 | hrpF  | 1154465 | HrpF protein             | GCCCGGACGAGTTG     | CGTGTGAGTGGTCAAA  | 62           | T3SS   |
| XAC0396 | hpaB  | 1154467 | HpaB protein             | GTCCTGTCAGCCAGAAC   | TGACGCCGCACTTGGT  | 53           | T3SS   |
| XAC0397 | hrpE  | 1154468 | HrpE protein             | AGCCGAGCGAAGCTTTCA | CCAGGTTGAGTCCAGATCATTT | 66           | T3SS   |
| XAC0398 | hrpD6 | 1154469 | HrpD6 protein            | ACCGATACGTCACCAAGAG | GACGCCACGACTTGGT  | 55           | T3SS   |
| XAC0399 | hrpD5 | 1154470 | HrpD5 protein            | GCCCTGAAGTGCGTTCGA | TCACGCGACGGCTTGGCT | 54           | T3SS   |
| XAC0400 | hpaA  | 1154471 | HpaA protein             | GAGCCACCCAACAGAAAAA | ACGGATCGCTTCCATCAA | 59           | T3SS   |
| XAC0401 | hrcS  | 1154472 | HrcS protein             | CGACGATCTAGTGCGAATT | CGACCACCGCAAGGA  | 68           | T3SS   |
| XAC0402 | hrcR  | 1154473 | Protein T3SS             | ACACGGGAAACCGCAA  | CGTCGCAATATCGCATCC | 58           | T3SS   |
| XAC0403 | hrcQ  | 1154474 | HrcQ protein             | GCAACGTGGAAGTGCA   | GACGGTAGTGGGACACC | 57           | T3SS   |
| XAC0404 | hpaP  | 1154475 | HpaP protein             | TGTCGAGTGGACAAATACC | CGAACCACGGCGATT | 58           | T3SS   |
| XAC0405 | hrcV  | 1154476 | HrcV protein             | GCGCAAGCTTGCGTCA   | CACATGGCGCATCACTT | 58           | T3SS   |
| XAC0406 | hrcU  | 1154477 | HrcU protein T3SS        | AGCCCGGCGTGCCTTTG | TAGTCGCGCAGCGGATTT | 51           | T3SS   |
| XAC0407 | hrpB1 | 1154478 | HrpB1 protein            | CTCGCGCCAGACTGAAG  | ACCGCGGTGGATGGAAC | 60           | T3SS   |
| XAC0408 | hrpB2 | 1154479 | HrpB2 protein            | CAGCGCAAGCTCAAGTGG | CGCCGAGCGTGACATT | 69           | T3SS   |
| XAC0409 | hrcJ  | 1154480 | HrcJ protein             | GAGCCGACGGCGCAATC  | TGTCGAGATCAAGCCATCTT | 54           | T3SS   |
| XAC0410 | hrpB4 | 1154481 | HrpB4 protein            | GACGACAACGGCGGATGA | GTCCGTCACGGCGTGA | 52           | T3SS   |
| XAC0411 | hrpB5 | 1154482 | Protein T3SS HrpB        | CGCAGCGTGCCTGGAATAG | AGCCGCGATCGTTCCC | 63           | T3SS   |
| XAC0412 | hrcN  | 1154483 | T3SS ATPase protein      | TGATGCGCACTGATTCTC | CGTGTGAGTGGGCTACT | 63           | T3SS   |
| XAC0413 | hrpB7 | 1154484 | HrpB7 protein            | CGCCGCGTGTGGAACAG  | GCTCGAGATCTGTCGATA | 60           | T3SS   |
| XAC0414 | hrcT  | 1154485 | HrcT protein             | ATTCAGCAGTCGACAGCAT | CACGGGATCGGAGATTT | 54           | T3SS   |
| XAC0415 | hrcC  | 1154486 | HrcC protein             | CAGATGCGGCTGATGTTG | CCCTGACCGGCTGGATTG | 59           | T3SS   |
| XAC0416 | hpa1  | 1154487 | Hpa1 protein monovalent cation/H+ antiporter subunit E Glucosyltransferase MdoH | CGACGAGCGCGCTAGT | TTCATGCACTCGGCTGGATTG | 57           | T3SS   |
| XAC0459 | phaE  | 1154530 | phaE Glucosyltransferase MdoH | CCGATGCGGTGAATGTTG | GCATACACAGACCCACATC | 86           | T3SS   |
| XAC0618 | hrpM  | 1154689 | hrpM Glucosyltransferase MdoH | CAGGGCCTGCACTGGATG | GCATACACAGACCCACATC | 86           | T3SS   |
| XAC1265 | hrpG  | 1155336 | HrpG protein             | CGCGCAAGCGCATGGTATT | GAAAGAGCGAGCAGCAGCATT | 54           | T3SS   |
| XAC1266 | hrpXct | 1155337 | HrpX protein             | GCCTACAGCTACATGATCAAT | TGCCGCGACTCTGGTGAA | 63           | T3SS   |
| XAC ID   | Gene | Gene ID | Product (NCBI Annotation) | Primers (Forward) | Primers (Reverse) | Amplicon (bp) | System               |
|----------|------|---------|--------------------------|-------------------|------------------|---------------|---------------------|
| XAC1520  | hrcA | 1155591 | Heat-inducible transcriptional repressor | AGTTCGCGTTTCGGAATAC | CGGTTCTGCACCTCGGT | 91            | T3SS                |
| XAC1994  | hrxX | 1156064 | HrpX-like protein | CTGGTCTGATGCGGACTTTCT | GCGTACTCTGTCACGATCAA | 90            | T3SS                |
| XAC2047  | phaE | 1156118 | PHA synthase subunit | AGCAGGGGTGATCAAGAGAAG | TTTCGAGGAGCGCCTTTT | 63            | T3SS                |
| XAC2606  | —    | 1156677 | Hypothetical protein | CCGGAGCCCATGCTGAGA | AGGGAGGCGTGAATCAT | 59            | H,T4SS,C            |
| XAC2611  | virB6| 1156683 | VirB6 protein | TGCAGCAGGGAGGATTAGGA | CGCGCGTGCAGTGAT | 56            | T4SS,C              |
| XAC2613  | —    | 1156684 | Hypothetical protein | CGAAGGGAGATGGAAGCTT | GCTCAGAAGATGCCGTGGA | 59            | H,T4SS,C            |
| XAC2614  | virB4| 1156685 | VirB4 protein | GAGTCGATGGTGGAGAA | TTTTCTGAGGCGGAAATCT | 53            | T4SS,C              |
| XAC2615  | virB3| 1156686 | VirB3 protein | GCGCCAGATGCATTTTGA | GCAGCCAGACGAAATGA | 54            | T4SS,C              |
| XAC2616  | virB2| 1156687 | VirB2 protein | TCACGCGATGCGAAAATG | CAGCAACGAAATGAA | 62            | T4SS,C              |
| XAC2617  | virB1| 1156688 | VirB1 protein | CGATGATGCTGTCGGTAT | CACCCAGGCTGCCAA | 57            | T4SS,C              |
| XAC2618  | virB10| 1156689 | VirB10 protein | CGCCTGCAAGGACACACC | CCTGCGGGCTGTCGTCA | 56            | T4SS,C              |
| XAC2619  | virB11| 1156690 | VirB11 protein | CCATTGAGCCTCCGAT | GAATCTCCAGGCGTGGT | 61            | T4SS,C              |
| XAC2620  | virB9| 1156691 | VirB9 protein | GTCAGCCGGACAGA | GCTCCGATCCAGGAA | 59            | T4SS,C              |
| XAC2621  | virB8| 1156692 | VirB8 protein | TCGCTATGGCAAGATGGTAT | CGGAGTGGGCTGCA | 61            | T4SS,C              |
| XAC2622  | —    | 1156693 | Hypothetical protein | GCGGAGATTCCAAATGCGT | TGGCCGACATGAAGGTTG | 59            | H,T4SS,C            |
| XAC2623  | virD4| 1156694 | VirD4 protein | TCGCTGGAATCCGAT | TCAATGCAACAGCGAA | 59            | T4SS,C              |
| XAC2922  | hprW | 1156993 | HprW protein | GGGTGGAACACACGGCAT | TGCCCTTACAGGGAAGTCT | 58            | T4SS,C              |
| XAC3122  | hprA | 1157193 | ATP-dependent RNA helicase | TGGCAGCCGCTATGCTG | GGACTGGGTCAGATCTCCACGTA | 76            | T3SS                |
| XACb0035 | —    | 1158522 | Hypothetical protein | CGAGACGAGAAGCCGCTTAA | TGCAGCAAGCAGAATG | 55            | H,T4SS,P            |
| XACb0036 | virB1| 1158523 | VirB1 protein | CGATGATGCTGTCGGTAT | CACCCAGGCTGCCAA | 57            | T4SS,C              |
| XACb0037 | virB11| 1158524 | VirB11 protein | CGATGATGCTGTCGGTAT | CACCCAGGCTGCCAA | 57            | T4SS,C              |
| XACb0038 | virB10| 1158525 | VirB10 protein | TCTCAGTGAACAAAGACCTGCGT | CGTGTGCTGTCGTGAT | 64            | T4SS,P              |
| XACb0039 | virB9| 1158526 | VirB9 protein | GTCCTAGGCGCGGACTT | GACTGGTTGTTGACGACATC | 58            | T4SS,P              |
| XACb0040 | virB8| 1158527 | VirB8 protein | CTCGAAACACAGCTGCA | AACAGACGAAACCCGAT | 60            | T4SS,P              |
| XACb0041 | virB6| 1158528 | VirB6 protein | GCGGACGGCTCGCTCATG | CGCGGACGCCAACAGAT | 55            | T4SS,P              |
| XACb0042 | —    | 1158529 | Hypothetical protein | CGGAGGCGGCTATT | GGAAGCGCAGTGCAGA | 52            | H,T4SS,P            |
| XAC ID | Gene | Gene ID | Product (NCBI Annotation) | Primers (Forward) | Primers (Reverse) | Amplicon (bp) | System |
|--------|------|---------|---------------------------|-------------------|-------------------|--------------|--------|
| XACb0043 | —    | 1158530 | Hypothetical protein      | TTCGGCGGCCCTCATCTC | AGGGCGACTTTGTTGAACTTTGTT | 58           | H,T4SS,P |
| XACb0044 | virB5 | 1158531 | VirB5 protein             | CAGGCCGCTACGAGAAG | GGGATGGGCAACAGGTCTT  | 57           | T4SS,P   |
| XACb0045 | virB4 | 1158532 | VirB4 protein             | GGAACAGGCAAAAGACGTCAAC | CCCGACAAAGGTTGAACGA | 57           | T4SS,P   |
| XACb0046 | virB3 | 1158533 | VirB3 protein             | TCTTTCGATGACTTATATTACC | CCCAAATGATCGGTGATT  | 59           | T4SS,P   |
| XACb0047 | virB2 | 1158534 | VirB2 protein             | CGATCTGGCGCTACCGTGTT | ATCGGCGCTCGGAAAGGAA | 56           | T4SS,P   |
| XACb0048 | —    | 1158535 | Hypothetical protein      | GGCGGATGTGCTTGAAAGC | CGCGTAAACGACCTCATAAC | 56           | H,T4SS,P |
| XACb0049 | —    | 1158536 | Hypothetical protein      | ACCACGGATCCTGGCAAAT | AGCAGCCCCCGTGAAACA  | 51           | H,T4SS,P |

This table provides primers sequences, ORF's identification ID, gene's name, and respective Xanthomonas citri subsp. citri secretory systems. T4SS: type IV secretion system; T3SS: type III secretion system; C: chromosomal copy; P: plasmid copy; H: hypothetical genes associated with the T4SS.
Figure 1: T3SS genes in *Xanthomonas citri* subsp. *citri* and their expression levels. Schematic representation of T3SS genes in *Xanthomonas citri* subsp. *citri* and their expression levels. (a) shows functional maps of T3SS genes on the chromosome. The expression levels of genes in the T3SS (b) are indicated. Solid green bars indicate *in vivo* expression with respect to their expression *in vitro* (black bars). Error bars indicate standard deviation of the replicates.

Figure 2: T4SS genes in *Xanthomonas citri* subsp. *citri* and their expression levels. Schematic representation of T4SS genes in *Xanthomonas citri* subsp. *citri* and their expression levels. (a) and (b) show functional maps of T4SS genes on the chromosome (a) and plasmid pXAC64 (b), respectively. The expression levels of genes in the T4SS (c) and ORFs localized near the *virB* genes (d) are indicated. Solid green bars indicate *in vivo* expression with respect to their expression *in vitro* (black bars). Error bars indicate standard deviation of the replicates.
Our qPCR data showed that *hpa1* was the most up-regulated gene during *Citrus sinensis* infection by *Xcc*, followed by *hrpD6* (Figure 1(b)). Conversely, *hrpA*, which encodes an important component of the T3SS pilus [39], was down-regulated in our study. Previously, *hrpA* mutants of *Pseudomonas syringae pv. tomato* DC3000 showed reduced accumulation of structural components of the *hrp* pilus [39]. Haapalainen et al. [40] showed that soluble plant cell signals induce the expression of *hrp/hrc* genes and specifically upregulate *hrpA*. Interestingly, they found that HrpA does not accumulate to high levels intracellularly, suggesting that some kind of feedback regulation takes place between secretion and production of HrpA, perhaps through degradation of intracellular HrpA. Our work shows that this may also be applicable to *hrpA* gene in *Xcc*: its mRNA may be degraded faster, just as happen with HrpA in *Pseudomonas syringae*.

4. Conclusions

Our finds confirm that the T3SS genes are induced in the successful infection of *Citrus sinensis* by *Xcc*, and therefore are excellent targets to help control or decrease the severity of citrus canker, whereas T4SS seems not to have any relation with virulence. On the other hand, our results show that T4SS is not induced under the same infection conditions, which could indicate that it is not necessary for infection but only for cell-to-cell communication.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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