Diffusible signal factor (DSF)-mediated quorum sensing modulates expression of diverse traits in Xanthomonas citri and responses of citrus plants to promote disease

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Abstract

Background: The gram-negative Xanthomonas genus contains a large group of economically important plant pathogens, which cause severe diseases on many crops worldwide. The diffusible signal factor (DSF) - mediated quorum sensing (QS) system coordinates expression of virulence factors in plant pathogenic Xanthomonas spp. However, the regulatory effects of this system during the Xanthomonas-plant interactions remain unclear from both the pathogen and host aspects.

Results: In this study, we investigated the in planta DSF- mediated QS regulon of X. citri subsp. citri (Xac), the causal agent of citrus canker. We also characterized the transcriptional responses of citrus plants to DSF-mediated Xac infection via comparing the gene expression patterns of citrus triggered by wild type Xac strain 306 with those triggered by its DSF- deficient (ΔrpfF) mutant using the dual RNA-seq approach. Comparative global transcript profiles of Xac strain 306 and the ΔrpfF mutant during host infection revealed that DSF- mediated QS specifically modulates bacterial adaptation, nutrition uptake and metabolisms, stress tolerance, virulence, and signal transduction to favor host infection. The transcriptional responses of citrus to DSF-mediated Xac infection are characterized by downregulation of photosynthesis genes and plant defense related genes, suggesting photosynthetically inactive reactions and repression of defense responses. Alterations of phytohormone metabolism and signaling pathways were also triggered by DSF-mediated Xac infection to benefit the pathogen.

Conclusions: Collectively, our findings provide new insight into the DSF- mediated QS regulation during plant-pathogen interactions and advance the understanding of traits used by Xanthomonas to promote infection on host plants.

Keywords: Xanthomonas, Diffusible signal factor (DSF), Quorum sensing, Citrus canker, Transcriptomic profiling

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Background

The genus *Xanthomonas* comprises a large group of gram-negative plant pathogenic bacteria that have considerable agricultural impact worldwide, and therefore, is an important model genus for studying the host-pathogen interactions [1, 2]. Successful infection and bacterial multiplication of *Xanthomonas* spp. in host tissues require coordinated expression of a combination of virulence factors. Key virulence factors of *Xanthomonas* spp. include, among others, the type III secretion system (T3SS) and its effectors [3, 4], bacterial polysaccharides such as the xanthan extracellular polysaccharides (EPS) and lipopolysaccharides (LPS) [5], and cell wall degrading enzymes [1]. Expression of these virulence factors is regulated by different extracellular stimuli via multiple coordinated regulatory systems, including cell-to-cell communication (quorum-sensing, QS) pathways, two-component systems and various transcriptional regulators [1].

The QS regulatory systems of *Xanthomonas* are mediated by molecules belonging to the diffusible signal factor (DSF) family [2, 6, 7]. The DSF-mediated QS has been studied most extensively in the crucifer pathogen *X. campestris* pv. *campestris* (Xcc). The synthesis and perception of the DSF signal, which was identified as cis-11-methyl-2-dodecenoic acid [8], require the *rpf* gene cluster (for regulation of pathogenicity factors), including *rpfB*, *rpfF* and *rpfGHC* [9, 10]. *RpfB* was initially thought to be involved in DSF biosynthesis, but it was later identified as a fatty Acyl-CoA ligase involved in the turnover of the DSF family of signals in *Xanthomonas* [11]. The *RpfF* protein, functioning as a putative enoyl-CoA hydratase, is responsible for the synthesis of DSF. *RpfC* and *RpfG* consist of a two-component system involved in DSF perception and signal transduction. *RpfC* is a hybrid sensor kinase and *RpfG* is a response regulator with a CheY-like receiver (REC) domain and an HD-GYP domain, capable of degrading the second messenger cyclic di-GMP [6, 10, 12, 13]. DSF can bind directly to the N-terminal, 22 amino acid-length sensor region of *RpfC* and activate *RpfC* autokinase activity to regulate QS and virulence in *Xcc* [14]. *RpfH* is a putative membrane protein with no known role in DSF signaling [10].

The contribution of DSF/Rpf regulatory system to virulence has been demonstrated in many members of Xanthomonas. For example, DSF signaling in *Xcc* influences the synthesis of a range of virulence factors including extracellular enzymes such as endoglucanase, protease, and endomannanase, and the xanthan EPS, as well as alterations in biofilm formation [6, 10, 15]. Specifically, the *RpfS*-dependent second DSF signaling pathway controls expression of genes involved in type IV secretion and chemotaxis and therefore affects bacterial motility, suggesting a role in the epiphytic phase of the *Xcc* disease cycle [16]. Similarly, the DSF-mediated QS has been shown involved in early attachment and *in planta* growth of *Xac* in the citrus host during the citrus canker disease cycle [17]. Recent report indicates that the DSF family in *Xcc* elicited plant innate immunity and this effect was suppressed through the secretion of the xanthan exopolysaccharide [18]. DSF also confers a fitness advantage to *Xcc* during interspecies competition [19].

Transcriptome profile, functional genomics, and proteomic analyses have significantly advanced the understanding of the DSF/Rpf regulatory network and its role in pathogenesis of *Xanthomonas*. Earlier studies have revealed that the *RpfC/RpfG* two-component system coordinately regulates the expression of various genes related to virulence via the cyclic di-GMP signaling that activates the transcriptional activators Clp and Zur in *Xcc* [6, 12, 13, 20]. These include the genes encoding extracellular enzymes, components of type II secretion system (T2SS), components of type III secretion system (T3SS), and the genes involved in EPS production. Comparative transcriptome studies using whole-genome microarray showed that the DSF/Rpf-mediated QS regulation in the citrus canker pathogen *X. citri* subsp. *citri* (*Xac*) is growth phase-dependent, and more genes in the exponential phase are differentially regulated by the *RpfC/RpfG* system compared with in the stationary phase [17]. The *RpfC/RpfG* system-regulated genes include diverse genes involved in chemotaxis and motility, flagellar biosynthesis, production of extracellular enzymes and adhesins, stress tolerance, regulation, transport, and detoxification [17]. There are also some unique genes controlled by *RpfF*, *RpfC* or *RpfG* alone, indicating the complexity of the QS pathway and the involvement of additional DSF signal perception and transduction mechanisms in *Xac* [17]. Interestingly, recent studies suggested additional signaling outputs from *RpfC* and an interaction of *RpfG* with a second unknown sensor [16, 21]. The authors found that DSF and *RpfC* also regulate expression of a number of genes encoding transcriptional regulator, hydrolase, protease and hypothetical proteins independently of *RpfG*, and *RpfC* regulates expression of genes involved in chemotaxis, signal transduction and protein export, independently of *RpfF* or *RpfC* [16, 21]. These studies also revealed that *RpfC* can recognize other unidentified environmental signals (in addition to DSF) [21] and the DSF signal can be recognized by a second sensor *RpfS*, a PAS domain-containing histidine kinase that regulates genes involved in type IV secretion and chemotaxis in a pathway independent of *RpfC* and *RpfG* [16]. Our knowledge of the protein(s)/regulator(s) acting downstream of *RpfS* in DSF signal transduction cascades remains limited. In
addition, the DSF/Rpf system controls three non-coding RNA (ncRNAs) that contribute to virulence in Xcc [21].

Comparative proteomic analysis revealed diverse regulatory effects of DSF/Rpf in Xcc on proteins involved in regulation, biosynthesis and intermediary metabolism, stress tolerance, and motility [22]. Similarly, mutation of the rpfF gene has a substantial impact on the proteome of X. oryzae pv. oryzae, affecting proteins involved in a range of functions including nitrogen transfer, protein folding, resistance to oxidative stress and flagellar synthesis [23]. Interestingly, for many of the proteins regulated by the DSF/Rpf system in Xcc, the alteration in abundance was not associated with alteration in transcript level, suggesting that both posttranscriptional regulation and post-translational turnover may occur [22].

Despite the extensive transcriptome analyses of the DSF/Rpf regulatory system in Xanthomonas spp., as stated above, most of which were performed using the bacterium grown under culture media conditions, and knowledge on regulatory effects of the DSF/Rpf system of Xanthomonas spp. during the interaction with host plants is still lacking. The actions of the elements involved in DSF signaling and the role of DSF signal transduction during plant infection remain to be determined from both the pathogen and host aspects. In the present study, we investigated the DSF/Rpf QS regulation in planta during Xac infection of citrus host. Metatranscriptome analysis of the compatible interaction between Xac and citrus was conducted using RNA-Seq to compare the global transcriptomes of wild-type and isogenic rpfF mutant (ΔrpfF) strains of Xac, as well as the citrus transcriptional patterns in response to their infection. This work provides a comprehensive picture of the genes and traits regulated by the DSF/Rpf QS in Xac in planta and host responses to the DSF-mediated infection.

**Methods**

**Bacterial strains and growth conditions**

*Xanthomonas citri* subsp. *citri* (*Xac*) wild type strain 306 [24] and its rpfF gene deletion (ΔrpfF) mutant strain [17] were grown at 28 °C with shaking at 200 rpm in nutrient broth (NB; Difco, Detroit, MI) containing rifamycin (50 μg/mL). Bacterial growth was measured in a spectrophotometer at 600 nm.

**Plant inoculations and sampling of infected leaves**

Plant inoculation was performed as described in our previous work [5]. Briefly, young (about 12-week-old) Duncan grapefruit (*Citrus paradisi* Macfad) plants were grown in potting medium/soil in a greenhouse at the Citrus Research and Education Center, Lake Alfred, FL, USA, and maintained at approximately 25–30 °C and a 55% relative humidity until the primary leaves were fully expanded but not fully matured. The bacterial inoculum cells were grown as described above. When the cells reached late-log phase (OD_{600} = 1.0; 5 × 10^{8} cfu/mL), they were collected by centrifugation at 4000 rpm for 15 min. The cell pellets were resuspended in sterilized 0.85% NaCl, washed, and resuspended in sterilized 0.85% NaCl to a final density of 5 × 10^{6} cfu/mL. To establish in planta populations, bacteria were introduced by infiltration into leaves using a needleless syringe. Infiltrated plants were maintained in the same greenhouse for canker symptom development. All plant inoculations included at least three leaves at a similar developmental stage from each plant, and ten replicate plants were inoculated for each strain. Time-course bacterial growth in *planta* was tested as described previously [5]. All the tests were repeated three times. Based on the progression of development of canker symptoms, the infected leaves were sampled at 5 days post inoculation (DPI) for RNA extraction. The inoculated leaf area was collected using a cork borer (leaf area, 1 cm²) and 10 leaf samples from each biological replicate (three replicates for each treatment) were pooled and immediately frozen in liquid nitrogen, and kept in −80 °C until process for RNA isolation.

**RNA extraction, library construction and Illumina RNA-seq**

Total RNA was extracted from leaf samples using RNeasy Plant Mini Kit (Qiagen, Valencia, CA) and contaminated DNA was removed by treatment with RNase-Free DNase Set (Qiagen, Valencia, CA). The quality and quantity of RNA samples were assessed using NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE), Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) and agarose gel electrophoresis. The total RNA was treated with DNase I (New England Biolabs, Ipswich, MA) prior to library construction. The rRNA of plant and bacteria was depleted using Ribo-Zero™ rRNA Removal Kits (Plant Leaf) and Ribo-Zero™ rRNA Removal Kits (Bacteria) respectively, according to the manufacturer's instructions (Epicenter Technologies, Madison, WI). Poly (A) + mRNA was purified using Agencourt RNAClean XP Kit (Beckman Coulter Life Sciences, Indianapolis, IN) and fragmented into short pieces. Using these short fragments as templates, first strand cDNA synthesis was conducted using random hexamer-primers and SuperScript™ II Reverse Transcriptase (Invitrogen, Waltham, MA), and the second-strand cDNA was synthesized using RNase H (Invitrogen, Waltham, MA) and DNA polymerase I (New England Biolabs, Ipswich, MA). After purification, end repair, and poly (A) tails add, the cDNA fragments were ligated to
sequencing adapters. Then fragments of an appropriate size were purified and amplified by PCR to produce the final library. Finally, the cDNA libraries were loaded onto the flow cell channels of an Illumina HiSeq TM 2000 platform for paired-end 90 bp × 2 sequencing at the Beijing Genomics Institute (BGI), Hongkong, China. Clean reads were obtained after removing reads containing adaptor sequences. The RNA reads have been deposited at NCBI under the bioproject No. PRJNA421992 with the SRA accession no. SRP126698.

Reads mapping and differential expression analysis
The clean reads were firstly aligned to the Xac strain 306 genome (https://www.ncbi.nlm.nih.gov/nuccore/AE008923.1/) [24] using bowtie2 [25] with default parameters. The in planta differential expressed genes between wild type Xac 306 and ΔrpfF mutant strains were identified using DESeq2 R package [26] with the following cutoffs: [fold change] ≥ 2 and ajust-P ≤ 0.05. After aligned to Xac strain 306 genome, the remaining reads from each sample were analyzed mainly following the tuxedo pipeline [27]. Briefly, the reads were aligned to the sweet orange genome [28] using Tophat (v2.0.13) [29], and the generated alignments were fed to Cufflinks (v2.2.1) for transcript assembly [30]. The assemblies were combined with the sweet orange annotations using the cuffmerge algorithm and then fed to the cuffdiff2 for differentially expressed gene calling. Only the genes with [fold change] ≥ 2, q-value ≤ 0.05 and FPKM ≥ 1 were considered as significantly differentially expressed genes (DEGs) between wild type strain infected and ΔrpfF mutant strain infected plants. The MapMan gene functional categories were assigned to the DEGs using Mercator [30, 31] and the differentially regulated bins were identified by using MapMan [32].

Functional annotation and classification
For citrus DEGs, the corresponding reference ID were obtained by blasting them to CitrusPLEX in plant expression database (PLEXdb, http://www.plexdb.org/plex.php?database=Citrus) [33]. Gene Ontology (GO) enrichment analysis of functional significance was applied to map all DEGs to terms in the agriGO (http://bioinfo.cau.edu.cn/agriGO/) database [34], looking for significantly enriched GO terms in DEGs. For bacterial DEGs, Clusters of Orthologous Groups (COG, https://www.ncbi.nlm.nih.gov/COG/) enrichment analysis was performed by comparing the prevalence of DEGs assigned to a specific COG category to the prevalence of genes in the whole genome assigned to that COG category with a Fisher's exact test.

Validation of RNA-seq results by qRT-PCR
To verify the RNA-seq result, qRT-PCR assays were conducted using the same set of RNA samples as for RNA-seq analysis. The aliquoted RNA sample (1 μg) used for RNA-seq was reverse transcribed using a QuantiTect Reverse Transcription kit with random hexamer primers (Qiagen, Valencia, CA) for two-step qRT-PCR. The gene specific primers (Additional file 1: Table S1) were designed to generate amplicons of 70 to 150 bp based on the DEGs sequences of citrus plant and Xac strain 306. qRT-PCR was conducted using QuantiTect SYBR Green PCR Kit (Qiagen, Valencia, CA) and the 7500 fast real-time PCR system (Applied Biosystems, Foster City, CA). Melting curve analysis of the PCR products was performed at the end of each PCR cycle to confirm the amplicon specificity. The housekeeping gene CgGAPDH [35] and gyrA [5] was used as plant and bacterial endogenous control, respectively. All reactions were repeated with three independent biological replicates and two technical replicates. The relative fold change in target gene expression was calculated by using the formula 2△△CT [36].

Statistical analysis
Quantitative data were expressed as mean ± S.E.M. Statistical differences were evaluated through t-test and the level of statistically significant difference was set at P<0.05. All statistics were conducted using SAS 9.1.3.

Results
Canker progression and symptoms in inoculated citrus plants
Duncan grapefruit (Citrus paradisi Macfadyen) seedlings were inoculated with Xac wild type strain 306 and its DSF deficient (ΔrpfF) mutant strain for the development of typical symptoms of citrus canker. The first visible symptom, a water soaking area of the inoculated leaf, was observed at 5 days post inoculation (DPI). Within 14 DPI, typical symptoms of the canker disease were recorded (Fig. 1a): inoculated areas were characterized with water soaking, and then exhibited hypertrophic, necrotic, erumpent lesions, as evidenced by the raised pustules. The ΔrpfF mutant produced weaker water soaking phenotypes compared to wild type strain 306 at 5 DPI under the tested conditions; and this becomes more evident at 7DPI and until 9 DPI (Fig. 1a). However, the bacterial populations of the ΔrpfF mutant in planta were not significantly lower than the wild type strain (Fig. 1b).

Sequencing the early citrus canker transcriptome
As the differences in the development of canker symptoms only were recorded in early stages of disease development (formation of water-soaking phenotypes) between
the wild type Xac and ΔrpfF mutant (Fig. 1a), we speculated that the DSF/Rpf QS play certain role(s) in early stages of the Xac-citrus compatible interaction. Therefore, the early canker transcriptome during the formation of water-soaking phenotypes was profiled at 5 DPI. A total of 227 million and 278 million paired-end reads for wild type Xac strain 306 infected and the ΔrpfF mutant infected plants were produced respectively (Additional file 2: Table S2). All reads were aligned against the Xac 306 genome [24]. For each RNA-seq library, 2.4–5.5% of the reads mapped to the Xac 306 reference. Then the remaining Xac strain 306-unaligned reads were mapped against the sweet orange (Citrus sinensis) genome [28], for the analysis of the citrus host transcriptome. A significant fraction of the Xac306-unaligned reads (63–68%) from both wild type Xac infected and ΔrpfF mutant infected libraries mapped to the sweet orange reference (Additional file 2: Table S2). Finally, of the 4374 Xac genes, 202 (4.5%) were determined as significantly differentially expressed genes (DEGs) [a minimum absolute value of a log2-fold change greater than 1 (equivalent to two-fold)] between wild type Xac 306 and ΔrpfF mutant strains in the conditions analyzed (Additional file 3: Table S3). Among them, 138 were upregulated in Xac wild type strain 306 compared to ΔrpfF mutant and 64 were downregulated. Of the 29,445 citrus (sweet orange) genes, 1946 genes were identified as significantly DEGs between wild type Xac 306 infected and ΔrpfF mutant strain infected plants, with 708 genes upregulated and 1238 downregulated in the wild type Xac 306 infected plants compared to ΔrpfF mutant strain infected plants (Additional file 4: Table S4).

Fig. 1 Citrus canker disease development in Xanthomonas citri inoculated Duncan grapefruit plants. a Representative leaves from ten replicates to show the development of canker symptoms on leaves inoculated with Xanthomonas citri subsp. citri wild type (Wt) strain 306 and its ΔrpfF mutant (M) with bacterial solutions (5 × 10^6 CFU/ml) by infiltration using needleless syringes and photographed at different days post inoculation (DPI). b Bacterial population growth in Duncan grapefruit leaves inoculated with bacteria (5 × 10^6 CFU/ml) at different days post inoculation. Error bars represent standard deviation. All the experiments were repeated three times.
To validate the gene expression values obtained by RNA-seq, the expression of 40 Xac genes and 33 citrus genes (Additional file 1: Table S1) in the RNA samples used in RNA-seq analysis were analyzed by qRT-PCR assays. A strong correlation ($R^2 = 0.9141$ for Xac gene expression; $R^2 = 0.9011$ for citrus gene expression) were observed between the data produced by the two approaches (Fig. 2a-b), demonstrating the reliability of the results obtained.

**Functionally categorizing of Xac genes regulated by the DSF/Rpf-mediated QS system during early stages of host infection**

The 202 DEGs of Xac were subject to functionally categorizing with enrichment analyses of clusters of orthologous groups (COGs). The results showed that overrepresented COGs terms were mostly related to ‘Carbohydrate transport and metabolism’ (39 members, 16.3%), ‘Amino acid transport and metabolism’ (29 members, 14.4%), ‘Inorganic ion transport and metabolism’ (21 members, 10.4%), and ‘Cell wall/membrane/envelope biogenesis’ (19 members, 9.41%) (Fig. 3). Other enriched terms included ‘Lipid transport and metabolism’, ‘Energy production and conversion’, ‘Post-translational modification, protein turnover, and chaperones’, ‘Signal transduction mechanisms’, ‘Transcription’, and ‘General function prediction only’. In addition, the genes annotated as hypothetical proteins were assigned to the ‘Function unknown’ group.

**DSF/Rpf-mediated QS regulates stress tolerance of Xac during early stages of host infection**

A total 12 genes encoding enzymes involved in detoxification and stress tolerance of Xac at early stages of host infection were differentially regulated by DSF/Rpf-mediated QS (Table 1). Of these, the genes coding for a putative arabinose efflux permease belonging to the Major Facilitator Superfamily (MFS) transporter for sugar/drug (araJ/XAC1363), for a drug resistance translocase (yieO/XAC2494), for an endoprotease (argC/XAC2992), and for trehalose biosynthesis (XAC0425 and XAC0429) were upregulated by ≥2-fold on average. Bacterial endoproteinases are able to degrade host defense proteins [37, 38], and trehalose protects bacterial cells from osmotic and oxidative stresses [39, 40]. The katE gene (XAC1211) encoding a catalase important for hydrogen peroxide tolerance in Xac [41], was also upregulated by 2-fold.

**DSF/Rpf-mediated QS is implicated in the regulation of nutrition utilization of Xac during early stages of host infection**

A significant portion of the Xac in planta transcriptome regulated by DSF/Rpf-mediated QS is dedicated to nutrition utilization (Fig. 3). Of the 39 genes involved in carbohydrate uptake and metabolism, 14 were positively regulated by DSF/Rpf-mediated QS, while 25 were negatively regulated (Table 2). The carbohydrate genes upregulated by DSF/Rpf-mediated QS included those encoding cellulose endoglucanase (egl/XAC0029 and engXCA/XAC0612), glycosyl transferase
(grtB/XAC1038/XAC2125, XAC3533, and ugt/XAC3921), glycosyl hydrolase (XAC3073), glycogen synthase (glgA /XAC0425 and glgY/XAC0429), glucose dehydrogenase (gcd/XAC1633/XAC3212), glucokinase (glk/XAC3120), and transporter (araJ/XAC1363 and yieO/XAC2494).

In contrast, the expressions of fruBK and fruA encoding components of a fructose-specific phosphoenolpyruvate (PEP): carbohydrate phosphotransferase system (PTS) were downregulated.

For the genes involved in uptake and metabolism of amino acids, most (23 out of 29) were downregulated by DSF/Rpf QS in planta, while a small portion (6/29) were upregulated (Table 2). Among the downregulated genes, some are involved in the biosynthesis of asparagine (asnB/XAC1433), tyrosine (phhA/XAC0174), glutamine (glnA/XAC0204 and glnB/XAC0205), glycine (pucG/XAC0300 and amaB/XAC0301), threonine (thrAB/XAC1820, XAC1821, and thrC/XAC1823), histidine (hisGDCBHAFI/XAC1828–1835), and biosynthesis of isoleucine, leucine, and valine (ilvCGM, tdcB, leuA/XAC3451–3455).

Over-presented in the up-regulated genes are those for biosynthesis of methionine (metE/XAC0306) and lysine (dapA/XAC2547), for a metallo-proteinase (XAC0465), and for glycine biosynthesis and cleavage (gcvP/XAC1214). Remarkably, the urea amidolyase and an allophanate hydrolase, which catalyze the release of ammonia from urea, showed distinctive expression levels (Log2Fold Change ≥5.9) upregulated by DSF/RPF QS in Xac during host infection.

Eleven differentially expressed genes were related to inorganic ion transport and metabolism in Xac during host infection (Table 2). Remarkably, the two genes (phoX/XAC1578 and oprO/XAC1579) encoding phosphate transporter proteins were upregulated by an average of 2.6-fold by DSF/Rpf QS during infection. The genes for siderophore biosynthesis (entF/XAC3922) and for iron storage protein in the bacterioferritin family (bfr/XAC1149) were upregulated two-fold or more (Table 3). Six genes encoding TonB-dependent outer-membrane
Table 2 List of genes involved in nutrient transport or metabolism in Xac regulated by DSF/Rpf-mediated QS during pathogenic process

| Locus tag | Gene name | Log2Fold Change (Wt/ΔrpfF) | Annotation/ Protein function |
|-----------|-----------|-----------------------------|-----------------------------|
| XAC0029   | egl       | 1.34                        | cellulase                   |
| XAC0425   | glgA      | 1.03                        | glycogen synthase           |
| XAC0429   | glgY      | 1.04                        | malt-o-oligosyltrehalose synthase |
| XAC0612   | engICA    | 1.53                        | cellulase                   |
| XAC1038   | gntB      | 1.12                        | glycosyl transferase        |
| XAC1363   | araJ      | 1.32                        | MFS transporter             |
| XAC1633   | gcd       | 2.06                        | glucose dehydrogenase       |
| XAC2125   | gntB      | 1.07                        | glycosyl transferase        |
| XAC2494   | yieO      | 1.29                        | drug resistance translocase |
| XAC3073   |          | 1.00                        | GH18 family, chitinase-like glycosyl hydrolase |
| XAC3120   | glk       | 1.36                        | glucokinase                 |
| XAC3212   | gcd       | 1.05                        | gluconolactone synthase     |
| XAC3533   |          | 1.23                        | Glucosyltransferase, GT2 family |
| XAC3921   | ugt       | 1.52                        | glucosyltransferase         |
| XAC2017   | lgtB      | −1.06                       | glycosyltransferase         |
| XAC2099   |          | −2.16                       | polysaccharide /chitin deacetylase |
| XAC0575   | ganB      | −1.98                       | arabinogalactan endo-1,4-beta-galactosidase |
| XAC1286   | abfA      | −1.09                       | alpha-L-arabinofuranosidase |
| XAC1308   | bga       | −1.18                       | beta-galactosidase          |
| XAC1309   | gaiA      | −1.49                       | arabinogalactan endo-1,4-beta-galactosidase |
| XAC1556   | fucP      | −1.43                       | glucose-galactose transporter |
| XAC1557   | scrK      | −1.49                       | fructokinase                |
| XAC1558   |          | −1.46                       | putative N-acetylglucosamine 2-epimerase |
| XAC1770   | celA      | −1.03                       | cellulase                   |
| XAC1771   |          | −1.02                       | sialic acid-specific 9-O-acetylerase |
| XAC1793   | celD      | −2.46                       | glucan 1,4-beta-glucosidase |
| XAC1794   | folK      | −2.38                       | sodium/glucose cotransport protein |
| XAC1812   | hmsF      | −1.72                       | HmsF protein /Polysaccharide deacetylase |
| XAC1813   | hmsH      | −2.06                       | HmsH protein /Substrate-specific transmembrane transporter |
| XAC2501   | fruB      | −1.73                       | multiphosphoryl transfer protein |
| XAC2502   | fruK      | −1.68                       | 1-phosphofructokinase       |
| XAC2503   | fruA      | −1.79                       | PTS system fructose-specific transporter subunit II |
| XAC3474   | citI      | −1.08                       | citrate carrier protein     |
| XAC3487   | cebR      | −2.20                       | transcriptional regulator   |
| XAC3489   | fyuA      | −1.49                       | TonB-dependent sucrose outer membrane transporter |

Table 2 List of genes involved in nutrient transport or metabolism in Xac regulated by DSF/Rpf-mediated QS during pathogenic process (Continued)

| Locus tag | Gene name | Log2Fold Change (Wt/ΔrpfF) | Annotation/ Protein function |
|-----------|-----------|-----------------------------|-----------------------------|
| XAC3490   |          | −1.22                       | amylosucrase or alpha amylase |
| XAC4195   | ndvB/celAP| −1.23                       | NdvB protein/ cellobionic acid phosphorylase |
| XAC4355   |          | −1.34                       | Glyco_hydro like             |
| XAC4361   | ttuB      | −1.51                       | MFS transporter              |

Amino acid transport and metabolism

| Locus tag | Gene name | Log2Fold Change (Wt/ΔrpfF) | Annotation/ Protein function |
|-----------|-----------|-----------------------------|-----------------------------|
| XAC0336   | metE      | 1.72                        | 5-methyltetrahydropteroyl triglutamate-methyltransferase |
| XAC0465   |          | 1.37                        | metalloproteinase           |
| XAC1214   | gcxP      | 1.09                        | glycine dehydrogenase       |
| XAC2547   | dapa      | 1.06                        | dihydridopicolinate synthetase |
| XAC4326   | uatA      | 6.50                        | urea amidolase              |
| XAC4327   | uatA      | 9.92                        | allophanate hydrolase       |
| XAC0174   | phxA      | −1.14                       | phenylalanine 4-monooxygenase |
| XAC0204   | glnA      | −1.39                       | glutamine synthetase        |
| XAC0205   | glnB      | −2.08                       | nitrogen regulatory protein P-II |
| XAC0206   | amtB      | −2.78                       | ammonium transporter        |
| XAC0300   | pucG      | −2.08                       | serine-pyruvate aminotransferase |
| XAC0301   | amaB      | −2.72                       | allantoate amidohydrolase   |
| XAC1433   | asnB      | −1.19                       | asparagine synthetase B     |
| XAC1820   | thrA      | −1.24                       | bifunctional aspartokinase I/ homoserine dehydrogenase I |
| XAC1821   | thrB      | −1.20                       | homoserine kinase           |
| XAC1823   | thrC      | −1.24                       | threonine synthase          |
| XAC1828   | hisG      | −2.32                       | ATP phosphoribosyltransferase |
| XAC1829   | hisD      | −2.02                       | histidinol dehydrogenase    |
| XAC1830   | hisC      | −1.94                       | histidinol-phosphate aminotransferase |
| XAC1831   | hisB      | −1.73                       | imidazole glycerol-phosphate dehydratase/phosphatase |
| XAC1832   | hisH      | −1.36                       | imidazole glycerol phosphate synthase subunit HisH |
| XAC1833   | hisA      | −1.61                       | 1-(5-phosphoribosyl)-5- imidazole-4-carboxamide isomerase |
| XAC1834   | hisF      | −1.51                       | imidazole glycerol phosphate synthase subunit HisF |
| XAC1835   | hisI      | −1.12                       | phosphoribosyl-AMP cyclohydrolase |
| XAC3451   | ilvC      | −2.15                       | ketol-acid reductoisomerase |
| XAC3452   | ilvG      | −1.69                       | acetolactate synthase 2 catalytic subunit |
| XAC3453   | ilvM      | −1.49                       | acetolactate synthase isozyme II small subunit |
| XAC3454   | tdcB      | −1.71                       | threonine dehydratase       |
| XAC3455   | leuA      | −1.22                       | 2-isopropylmalate synthase  |

Lipid transport and metabolism

| Locus tag | Gene name | Log2Fold Change (Wt/ΔrpfF) | Annotation/ Protein function |
|-----------|-----------|-----------------------------|-----------------------------|
| XAC1826   |          | −1.09                       | sialic acid-specific 9-O-acetylerase |
| XAC1793   | cellD    | −2.46                       | glucan 1,4-beta-glucosidase |
| XAC1794   | folK     | −2.38                       | sodium/glucose cotransport protein |
| XAC1812   | hmsF     | −1.72                       | HmsF protein /Polysaccharide deacetylase |
| XAC1813   | hmsH     | −2.06                       | HmsH protein /Substrate-specific transmembrane transporter |
| XAC2501   | fruB     | −1.73                       | multiphosphoryl transfer protein |
| XAC2502   | fruK     | −1.68                       | 1-phosphofructokinase       |
| XAC2503   | fruA     | −1.79                       | PTS system fructose-specific transporter subunit II |
| XAC3474   | citI     | −1.08                       | citrate carrier protein     |
| XAC3487   | cebR     | −2.20                       | transcriptional regulator   |
| XAC3489   | fyuA     | −1.49                       | TonB-dependent sucrose outer membrane transporter |
Table 2 List of genes involved in nutrient transport or metabolism in Xac regulated by DSF/Rpf-mediated QS during pathogenic process (Continued)

| Locus tag | Gene name | Log-Fold Change (Wt/ΔrpfF) | Annotation/ Protein function |
|-----------|-----------|-----------------------------|------------------------------|
| XAC0159   | estA1     | 1.15                        | carboxylesterase type B     |
| XAC1037   | mmsB      | 1.03                        | 3-hydroxyisobutyrate dehydrogenase |
| XAC0375   | aea       | −1.42                       | lipase                      |
| XAC2012   | fadA      | −1.25                       | acetyl-CoA acetyltransferase |
| XAC2013   | fadB      | −1.66                       | 3-hydroxyacyl-CoA dehydrogenase |
| XAC3300   | estA      | −1.10                       | esterase                    |
| XAC3486   | fabG      | −3.14                       | 3-ketoacyl-ACP reductase    |
| XAC3959   | −1.69     | Acyl-CoA delta-9-desaturase  |

Inorganic ion transport and metabolism

| Locus tag | Gene name | Log-Fold Change (Wt/ΔrpfF) | Annotation/ Protein function |
|-----------|-----------|-----------------------------|------------------------------|
| XAC1578   | phaX      | 1.34                        | phosphate-binding protein   |
| XAC1579   | oprO      | 1.50                        | polyphosphate-selective porin O |
| XAC0296   | vanB      | −3.94                       | vanillate O-demethylase oxidoeductase |
| XAC3011   | vanA      | −3.07                       | vanillate O-demethylase oxygenase |
| XAC0742   | cirA      | −1.45                       | RcnB containing protein     |
| XAC0999   | cirA      | −1.04                       | colicin I receptor          |
| XAC3168   | bfeA      | −1.55                       | ferric enterobactin receptor |
| XAC3169   | bfeA      | −1.17                       | ferric enterobactin receptor |
| XAC3472   | oprO      | −1.82                       | polyphosphate-selective porin O |
| XAC3484   | oprO      | −2.90                       | porin                       |

Genes for signal transducers and/or transcriptional regulators regulated by DSF/Rpf-mediated QS in Xac during early stages of host infection

The expression of 12 genes coding for signal transducers and/or transcriptional regulators in Xac were differentially regulated by DSF/Rpf-mediated QS (Table 4). Of these, two genes were upregulated and 10 genes were downregulated. The two upregulated genes were XAC1328 and XAC3927, encoding a putative CheY-like superfamily protein and serine/threonine protein kinase respectively, both are of signal transducer activity.

Among those genes downregulated were the two genes ntrB (XAC0207) and ntrC (XAC0208) encoding the NtrB/C two-component system, which interacts with the RpfC/G system responding to DSF signal to regulate sigma54-dependent promoters in vitro [44]. In addition, the two-component sensor genes tctE (XAC3482) and XAC3720, the transcriptional regulator genes acoR (XAC0654), tetR (XAC2014), icsR (XAC 2934), and cebR (XAC3487), the AbrB ambiactive repressor and activator (XAC1883), and the Trp operon repressor gene (trpR/ XAC1827) were downregulated by the DSF/Rpf1-mediated QS. The homologues of these signal transduction and transcription factors constitute regulators of virulence and adaptation factors in many bacteria, including the human bacterial pathogens enterotoxigenic E. coli [45] and P. aeruginosa [46], and the model organism Bacillus subtilis strain 168 [47]. For example, the IscR transcriptional repressor in E. coli negatively controls the type I fimbriae colonization factor synthesis and biofilm formation in response to both iron limitation and oxidative stress [45]. The trp repressor negatively regulates expression of genes involved in tryptophan biosynthesis, transport, and metabolism in response to intracellular levels of tryptophan, but also regulates transcription initiation in several other operons related to tryptophan metabolism that are important for expression of virulence factors in E. coli and P.
aeruginosa [46]. Thus, they might function as regulators of virulence and adaptation factors in Xac by modulating biofilm formation and adhesion factor production, which are crucial for attachment and colonization of the tissues and for consequent invasion [17].

**Putative function of the hypothetical protein encoding genes within the DSF/Rpf-mediated QS regulon in planta**

BLASTx analysis showed that 39 of the 44 genes within the DSF/Rpf QS regulon encoding hypothetical proteins had significant similarities only to sequences in bacteria within the *Xanthomonas* genus. Based on sequence similarity and conserved domain detected, we defined putative functions for 24 of the 44 genes, which are potentially involved in bacterial adaptation and pathogenesis (Table 5). Some of these genes encode proteins with recognized roles in bacterial pathogenesis, such as members of the cell surface adhesion protein families (XAC3546) and chemotaxis protein families (XAC3753 and XAC3754). Interestingly, the genes encoding stress-induced protein (XAC2156) and Ferritin-like di-iron-carboxylate protein (XAC2155) were upregulated by DSF/Rpf–mediated QS and possibly involved in the

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**Table 4** Summary of Xac DEGs coding for signal transduction and transcriptional factors regulated by DSF/Rpf-mediated QS during pathogenic process

| Locus tag | Gene name | Log2Fold Change (Wt/ΔrpfF) | Annotation/ Protein function |
|-----------|-----------|-----------------------------|------------------------------|
| XAC1328   |           | 1.07                        | CheY-like protein superfamily |
| XAC3927   |           | 1.04                        | serine/threonine protein kinase |
| XAC0207   | ntrB      | −1.28                       | two-component system sensor protein |
| XAC0208   | ntrC      | −1.21                       | two-component system regulatory protein |
| XAC0654   | acoR      | −1.27                       | transcriptional regulator AcoR |
| XAC1827   |           | −2.41                       | hypothetical protein/ Trp repressor protein (represses transcription of the Trp operon) |
| XAC1883   |           | −1.00                       | hypothetical protein/ AbrB domain containing transcriptional regulator |
| XAC2014   |           | −1.29                       | TetR family transcriptional regulator |
| XAC2934   | iscR      | −1.02                       | hypothetical protein/ Iron-sulfur cluster regulator IscR (Fe-S assembly SUF system transcriptional regulator) |
| XAC3482   | tctE      | −1.02                       | two-component system sensor protein |
| XAC3487   | cebR      | −2.20                       | transcriptional regulator |
| XAC3720   |           | −1.18                       | hypothetical protein/ putative two-component system sensor kinase |

**Table 5** Summary of Xac DEGs encoding hypothetical proteins regulated by DSF/Rpf-mediated QS during pathogenic process

| Locus tag | Log2Fold Change (Wt/ΔrpfF) | Homologue [Bacterial species] | Identity (%) |
|-----------|-----------------------------|------------------------------|--------------|
| XAC2155   | 1.36                        | ferritin-like domain-containing protein [Xanthomonas group] | 99 |
| XAC2156   | 1.97                        | stress-induced protein [X. phaseoli] | 98 |
| XAC3073   | 1.01                        | GH18 Chitinase-like glycosyl hydroxase [X. citri] | 99 |
| XAC3533   | 1.23                        | glycosyltransferase, GT2 family [X. axonopodis] | 97 |
| XAC3546   | 1.29                        | autotransporter adhesion protein [X. citri] | 99 |
| XAC2095   | −1.64                       | 5-hydroxysousurate hydrolase [X. citri] | 98 |
| XAC2097   | −2.93                       | 2-oxo-4-hydroxy-4-carboxy-5-ureidoimidazoline decarboxylase [X. citri] | 99 |
| XAC2098   | −1.84                       | Nuclear transport factor 2 (NTF2-like) superfamily [X. axonopodis] | 99 |
| XAC2051   | −1.22                       | FUSC-like inner membrane protein (fusaric acid resistance) [X. citri] | 98 |
| XAC1397   | −2.05                       | Alginate export domain containing protein [X. axonopodis] | 99 |
| XAC1471   | −1.12                       | Glycine zipper 2TM domain containing protein [X. citri] | 98 |
| XAC1827   | −2.41                       | Trp repressor protein [Xanthomonas group] | 99 |
| XAC1883   | −1.00                       | AbrB domain containing transcriptional regulator [X. citri] | 99 |
| XAC1884   | −1.26                       | PIN (PilT N terminus) domain-containing protein [X. citri] | 99 |
| XAC2821   | −1.02                       | Crotonase/Enoyl-Coenzyme A (CoA) hydratase [Xanthomonas group] | 99 |
| XAC2934   | −1.02                       | Fe-S assembly SUF system transcriptional regulator [X. citri] | 99 |
| XAC3085   | −1.06                       | putative type III secretion system effector protein [Xanthomonas group] | 99 |
| XAC3439   | −1.16                       | putative secreted protein [Xanthomonas group] | 99 |
| XAC3506   | −1.67                       | Cellulose belonging to glycosyl hydrolase family 5 [X. citri] | 98 |
| XAC3507   | −1.99                       | Cel5 cellulose; Glycosyl hydrolase 12 superfamily [Xanthomonas group] | 98 |
| XAC3720   | −1.17                       | putative two-component system sensor kinase [Xanthomonas group] | 99 |
| XAC3753   | −1.22                       | putative chemotaxis membrane protein [Xanthomonas group] | 99 |
| XAC3754   | −1.01                       | putative chemotaxis membrane protein [Xanthomonas group] | 99 |
| XAC3856   | −1.19                       | calcium-binding protein, EFh Superfamily [X. citri] | 99 |
| XAC4219   | −1.09                       | Lipid-binding SYLF domain containing protein [Xanthomonas group] | 99 |
adaptation of Xac to the host environment. The genes encoding putative GH18 chitinase-like glycosyl hydrolase (XAC3073) and GT2 family glycosyltransferase (XAC3533) were also upregulated, involved in carbohydrate transport and metabolism. In contrast, the gene XAC3085 encoding a putative T3SS effector protein was downregulated, with an unknown function in Xac-citrus interaction.

Comparison of the DSF/Rpf-mediated QS regulons in planta and in vitro

Our previous work identified 180 genes constituting the DSF/Rpf regulon of Xac grown in culture medium in the exponential and/or stationary growth phase [17]. Among those, a set of 31 genes were overlapping with the in planta DSF/Rpf regulon, 26 of which showed similar trends in alteration of expression between the two environmental conditions (Additional file 5: Table S5). Specifically, a subset of 20 genes were identified in the DSF/Rpf regulon of Xac in the exponential growth phase, 25 genes were identified in the DSF/Rpf regulon of Xac in the stationary growth phase, and 14 genes were identified in both regulons. These genes were primarily involved in energy metabolism (carbohydrate transport and metabolism), protein fate and protein synthesis (amino acid transport and metabolism or post-translational modification), and signal transduction or transcriptional regulation, and some encode hypothetical proteins with unknown functions.

Overview of citrus transcriptional responses to DSF/Rpf-mediated Xac infection

Global analyses of the citrus transcripts in response to DSF/Rpf-mediated Xac infection revealed that the protein families related to stress responses, signaling pathways, hormone metabolism, and cell wall modification were over-represented according to the gene ontology (GO) analysis (Fig. 4). Individual gene responses in metabolic pathways were visualized using the MapMan tool (Fig. 5). Remarkable downregulation was observed for many genes related to photosynthesis, secondary metabolism, and plant defense response.

DSF/Rpf-mediated Xac infection represses photosynthesis in citrus

The expression levels of nine genes involved in photosynthesis decreased significantly in wild type Xac strain 306 infected leaf tissues, compared with the ΔrpfF mutant infected leaf tissues (Table 6). Transcripts for photosystem II oxygen-evolving enhancer protein PsbO (Cs7g03508) and photosystem II 22 kDa protein PsbS (Cs3g19650) were less abundant in wild type Xac infected leaves. Three transcripts encoding subunits of photosystem I also decreased in wild type Xac infected leaves, including photosystem I reaction center subunit II (Psad), VI-2 (Psah), and O subunit (Psao). In addition, the genes for photosynthetic electron transport protein plastocyanin (PetE) and for an ATP synthase subunit (the F-type H+ - transporting ATPase subunit gamma, Cs4g10260) were downregulated in wild type Xac infected leaves (Table 6). These results are in agreement with the notion that Xac is biotrophic during early stages of host infection [48, 49] and that biotrophic pathogen infection generally represses photosynthesis in host plants [50].

Alterations of hormone metabolisms in citrus responding to DSF/Rpf-mediated Xac infection

Significant transcriptional changes in response to DSF/Rpf-mediated Xac infection were observed for a group of genes related to plant hormone biosynthesis, transport, metabolism, and associated signal transduction (Table 7). Transcripts for auxin biosynthesis-related enzymes and auxin-responsive proteins, including indole-3-acetate beta-D-glucosyltransferase (IAGLU), UDP-glucosyltransferase (UGT74E2), and SAUR (small auxin-up RNA)-like auxin-responsive protein, were more abundant, while genes for the PIN or PIN-LIKES class of auxin transporters were downregulated in wild type Xac infected leaves. The gene Cs2g03270 encoding a 9-cis-epoxycarotenoid dioxygenase, a key enzyme for abscisic acid (ABA) biosynthesis [51], was downregulated in wild type Xac infected leaves, and genes for ABA-responsive (ABR) proteins were upregulated in wild type Xac infected leaves. Three genes involved in cytokinin biosynthesis (cytokinin synthase, isopentenyltransferase (IPT), and UDP-Glycosyltransferase superfamily protein) were downregulated in wild type Xac infected leaves, while two genes involved in cytokinin metabolic process (UDP-glucosyltransferase 85A5 (UGT85A5) and DON-Glucosyltransferase) were upregulated in wild type Xac infected leaves.

A total of 17 genes encoding the ethylene response factor (ERF) transcription factors were differentially expressed in wild type Xac infected leaves compared to ΔrpfF mutant infected leaves (Table 7). In particular, the transcripts for ERF1 and RAP2.1 were more abundant in wild type Xac infected leaves, while transcripts for EREBP-3, ERF-4, ERF-6, ERF104, and for an ethylene-regulated nuclear protein (ERT2) were less abundant in wild type Xac infected leaves. One gene (Cs4g05190) involved in ethylene biosynthesis was upregulated in wild type Xac infected leaves. Two genes for gibberellic acid (GA) biosynthesis (the CYP701A cytochrome p450 family protein) and GA inactivation (GA2OX: gibberellin 2-oxidase) [52] were downregulated in wild type Xac infected leaves (Table 7). Three genes involved in the GA response were also downregulated in wild type Xac
infected leaves, including those GAST-like (gibberellic acid stimulated transcript-like) and ARM repeat superfamily proteins.

Three genes involved in jasmonic acid (JA) biosynthesis or metabolisms were upregulated in wild type Xac infected leaves compared to ΔrpfF mutant infected leaves (Table 7). These included the gene encoding 12-oxophytodienoic acid reductases (OPR) (orange1.1 t03726) and the gene encoding a FMN-containing oxidoreductases (orange1.1 t03729) (for JA biosynthesis), and a S-adenosyl-L-methionine:jasmonic acid carboxyl methyltransferase (JMT) that catalyzes the formation of

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**Fig. 4** Gene Ontology classification of citrus differentially expressed genes (DEGs) response to the DSF/Rpf-mediated *Xanthomonas citri* subsp. *citri* infection. **a** Pie diagram depicting the relative abundance of each category of DEGs. The category was presented by functional classification followed by the corresponding percentage. **b** Column chart showing the relative abundance of the three main categories of DEGs: biological process, cellular component, and molecular function.

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**Fig. 5** Display of citrus differentially expressed genes (DEGs) response to the DSF/Rpf-mediated *Xanthomonas citri* subsp. *citri* infection that are involved in different metabolic pathways (a) or biotic/abiotic stress responses (b). The log2 fold change of gene expression (ΔrpfF-inoculated plants versus wild type *Xanthomonas citri* subsp. *citri* -inoculated plants) was analyzed using MapMan. Each square represents an individual gene within a category. Small squares colored in red and blue represent genes in infected plants that were up- and down-regulated by DSF/Rpf – mediated Xac infection, respectively. A false color scale was used and all the values were given on a log2 scale. The color saturates at a 4-fold change (i.e. log2 ratio = 2 or −2). A significant downregulation was observed for many genes that are involved in photosynthesis, secondary metabolism, or response to biotic stress including genes for signaling, hormone metabolisms, and plant defense responses.
methyl jasmonate (MeJA) from JA (Cs7g31430) [53]. In contrast, two genes (orangel.1 t03773 and orange1.1 t04376) encoding the chloroplast lipoxygenases required for wound-induced JA accumulation in Arabidopsis were downregulated in wild type Xac infected leaves. Three genes involved in SA metabolisms were differentially expressed in wild type Xac infected leaves compared to ΔrpfF mutant infected leaves (Table 7). Notably, a gene (Cs1g23160) encoding the methyl esterase 1 (MES1) with methyl salicylate (MeSA) esterase activity of hydrolyzing MeSA to SA in planta [54], was upregulated in wild type Xac infected leaves. In addition, two genes (Cs2g28310 and Cs6g18050) encoding S-adenosyl-L-methionine-dependent methyl transferases superfamily proteins involved in SA metabolic process were downregulated in wild type Xac infected leaves. Furthermore, four genes involved in brassinosteroid (BR) biosynthesis or responses were repressed in wild type Xac infected leaves. They are a cycloartenol synthase 1 (CAS1) and a C-8 sterol isomerase involved in the biosynthesis of BR, and two leucine-rich receptor-like protein kinase family proteins involved in BR signaling pathways [55] (Table 7).

### Table 6 Summary of citrus DEGs genes involved in photosynthesis

| ID          | Gene name (Locus) | Log2Fold Change (Wt/ΔrpfF) | Annotation/ Protein function |
|-------------|-------------------|-----------------------------|------------------------------|
| XLOC_017330 | psbQ (Cs7g03580) | −1.36                       | photosystem II oxygen-evolving enhancer protein |
| XLOC_008489 | psbS (Cs3g19650) | −2.10                       | photosystem II 22 kDa protein |
| XLOC_014472 | psaD (Cs5g31180) | −1.18                       | photosystem I reaction center subunit II |
| XLOC_002098 | psaH (Cs1g15170) | −1.14                       | photosystem I reaction center subunit VI-2 |
| XLOC_015536 | psaO (Cs6g12390) | −1.13                       | photosystem I subunit O |
| XLOC_008847 | petE (Cs3g26730) | −1.22                       | photosynthetic electron transport protein plastocyanin |
| XLOC_004226 | Cs2g26640         | −1.27                       | GLK2 transcription factor, regulating the expression of photosynthetic apparatus |
| XLOC_010577 | atpA (Cs4g10260) | −1.07                       | F-type H + transporting ATPase subunit gamma |
| XLOC_001762 | psaN (Cs1g09130) | 1.23                        | photosystem I reaction center subunit N |

### Citrus defense responses to DSF/Rpf-mediated Xac infection

Of the 1946 citrus DEGs between wild type Xac infected - and ΔrpfF mutant infected - libraries, 102 genes (5.4%) were identified to be involved in plant defense responses, with 32 genes upregulated and 70 genes downregulated by DSF/Rpf-mediated Xac infection (Additional file 6: Table S6; Table 8). Remarkably, 34 genes encoding plant immune receptor-like proteins or receptor-like kinases were downregulated. Eight genes encoding transcription regulators were downregulated, including three WRKY transcription factors- encoding genes (one for WRKY 4 and two for WRKY 53). In addition, four genes encoding pathogenesis-related (PR) family proteins were downregulated, including the genes encoding members of the PR-5 (thraumin) and PR-6 (protease inhibitor) subfamily (Additional file 6: Table S6). Three Kunitz protease inhibitors encoding genes were also downregulated, which were suggested to modulate programmed cell death in in Arabidopsis during plant–pathogen interactions [56]. Other downregulated genes included five genes encoding NB-ARC (nucleotide-binding adaptor shared by Apaf-1, resistance proteins, and CED-4) domain-containing disease resistance proteins [57], and three genes encoding MYB transcription factor family proteins, which are involved in various plant biological processes including defense responses [58].

Among the 32 genes upregulated by DSF/Rpf-mediated Xac infection, three genes encode WRKY transcription factors, including WRKY18, WRKY22, and WRKY54 (Table 8). Interestingly, in Arabidopsis, AtWRKY18 alone with AtWRKY40 and AtWRKY60, act as negative regulators of defense signaling [59]. Other upregulated genes include two genes coding for the MYB transcription factors, three genes for the PR family proteins including one PR-5 and two PR-6, four genes for the NB-LRR family receptors, five genes for wound-responsive or ΔrpfF-induced proteins, and a few others for disease resistance responsive proteins and stress responsive proteins (Additional file 6: Table S6).
Table 7 Summary of citrus DEGs genes involved in plant hormone metabolisms

| ID         | Locus      | Log 2Fold Change (Wt/ΔrpfF) | Annotation/ Protein function                                                                 |
|------------|------------|-----------------------------|------------------------------------------------------------------------------------------------|
| Auxin biosynthesis, metabolism, and signaling |            |                             |                                                                                                 |
| XLOC_012174 | Cs5g20410  | 1.22                        | Indole-3-acetate beta-D-glucosyltransferase (IAGLU)                                           |
| XLOC_031022 | –          | 1.38                        | Indole-3-acetate beta-D-glucosyltransferase (IAGLU)                                           |
| XLOC_005577 | Cs2g23750  | 2.45                        | UDP-glucosyltransferase acting on IBA (indole-3-butyric acid), affects auxin homeostasis       |
| XLOC_029081 | orange1.1  | 1.65                        | SAUR-like auxin-responsive protein family                                                    |
| XLOC_003150 | Cs2g06290  | 1.00                        | Aluminium induced protein with YGL and LRDR motifs, auxin-responsive                           |
| XLOC_015754 | Cs6g17000  | −1.61                       | Probable auxin efflux carrier component 1c (PIN1-c)                                          |
| XLOC_020295 | Cs7g31320  | −1.19                       | Auxin transporter-like protein 1 (PIN-like protein 1)                                       |
| XLOC_008042 | Cs3g10670  | −1.28                       | NAD(P)-linked oxidoreductase superfamily protein, auxin regulated                             |
| Abscisic acid (ABA) -related genes |            |                             |                                                                                                 |
| XLOC_004564 | Cs2g03270  | −1.21                       | 9-cis-epoxycarotenoid dioxygenase for ABA biosynthesis                                       |
| XLOC_004925 | Cs2g10990  | −1.71                       | UDP glycosyltransferase (UGT) for ABA biosynthesis                                            |
| XLOC_017286 | Cs7g02850  | 2.07                        | GRAM domain-containing protein, ABA-responsive protein-related                                |
| XLOC_017832 | Cs7g13470  | 2.64                        | GRAM domain-containing protein, ABA-responsive protein-related                                |
| XLOC_012807 | Cs5g32930  | 1.29                        | membrane-bound protein (Arabidopsis thaliana TSPO-related), induced by ABA                   |
| Ethylene - related genes |            |                             |                                                                                                 |
| XLOC_01327  | Cs4g05190  | 1.48                        | flavanone 3 hydroxylase, 2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein, involved in ethylene synthesis |
| XLOC_004668 | Cs2g05280  | 1.08                        | ERF (ethylene response factor) subfamily B-3 of ERF/AP2 transcription factor family (ERF1) |
| XLOC_014405 | Cs5g29870  | 1.86                        | ERF (ethylene response factor) subfamily B-3 of ERF/AP2 transcription factor family (ERF1) |
| XLOC_024633 | –          | 1.36                        | ERF (ethylene response factor) subfamily B-3 of                                              |

Table 7 Summary of citrus DEGs genes involved in plant hormone metabolisms (Continued)

| ID         | Locus      | Log 2Fold Change (Wt/ΔrpfF) | Annotation/ Protein function                                                                 |
|------------|------------|-----------------------------|------------------------------------------------------------------------------------------------|
| XLOC_007284 | Cs3g23270  | 1.79                        | DREB subfamily A-5 of ERF/AP2 transcription factor family (RAP2.1)                          |
| XLOC_005573 | Cs2g23660  | −1.31                       | Ethylene-responsive transcription factor 4 (Ethylene-responsive element-binding factor 4 homolog) (EREBP-3) |
| XLOC_003119 | Cs2g05620  | −1.32                       | Ethylene-responsive transcription factor 4 (Ethylene-responsive element-binding factor 4 homolog) (EREBP-3) |
| XLOC_001696 | Cs1g07950  | −1.17                       | ERF (ethylene response factor) subfamily B-1 of ERF/AP2 transcription factor family (ERF-4) |
| XLOC_001450 | Cs1g03280  | −1.07                       | ERF (ethylene response factor) subfamily B-3 of ERF/AP2 transcription factor family (ERF13) |
| XLOC_014725 | –          | −2.28                       | ERF (ethylene response factor) subfamily B-3 of ERF/AP2 transcription factor family (ERF-6) |
| XLOC_024283 | Cs9g13620  | −2.24                       | ERF (ethylene response factor) subfamily B-3 of ERF/AP2 transcription factor family (ERF104) |
| XLOC_023353 | Cs9g13610  | −2.04                       | ERF (ethylene response factor) subfamily B-3 of ERF/AP2 transcription factor family (ERF104) |
| XLOC_003353 | Cs2g09980  | −1.39                       | Ethylene-responsive nuclear protein / ethylene-regulated nuclear protein (ERT2)              |
| XLOC_028605 | orange1.1  | −1.38                       | Adenine nucleotide alpha hydrodolases-like superfamily protein, involved in response to stress |

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related to lignin biosynthesis [61]; and, histological analyses revealed an increased lignin deposition and the existence of cell wall reinforcement in Xac infected tissues [62]. Remarkably, 12 genes encoding cell-wall-modifying enzymes, including expansins, endoglucanases, glycosyl transferases, and xyloglucan endotransglycosylases/hydrolases, were upregulated by DSF/Rpf-mediated Xac infection (Additional file 8: Table S8). Nine genes encoding protein products involved in the synthesis of cell wall precursors were also upregulated. These results implied a more pronounced effect on cell wall modification upon

| ID          | Locus | Log Fold Change | Annotation/ Protein function                                                                 |
|-------------|-------|-----------------|------------------------------------------------------------------------------------------------|
| Cytokinin - related genes                                                                                                      |
| XLOC_023917 | Cs9g06010 | −1.51          | cytokinin synthase for cytokinin biosynthesis                                                |
| XLOC_003491 | Cs2g12620 | −1.14          | putative adenylate isopentenyltransferase (IPT), involved in cytokinin biosynthesis          |
| XLOC_008154 | Cs3g12960 | −1.59          | UDP-Glycosyltransferase superfamily protein, involved in cytokinin biosynthesis               |
| XLOC_030591 | orange1.1 | 1.01           | UDP-glucosyltransferase 85AS (UGT85AS), involved in cytokinin metabolic process               |
| XLOC_030963 | −      | 1.47           | DON-Glucosyltransferase, UDP-Glucosyl transferase superfamily protein, involved in cytokinin   |
| Gibberellin (GA)- related genes                                                                                               |
| XLOC_019477 | Cs7g14940 | −1.17          | gibberellin 2-oxidase (GA2OX), involved in gibberellin metabolic process                     |
| XLOC_028715 | orange1.1 | 1.35           | 12-oxophytodienoic acid reductases, involved in JA biosynthesis                            |
| XLOC_029630 | orange1.1 | 1.64           | FMN-containing oxidoreductases, involved in JA biosynthesis                               |
| XLOC_020598 | Cs7g31430 | 1.05           | S-adenosyl-L-methionine: jasmonic acid carboxyl methyltransferase (JMT), involved in JA       |
| Brassinosteroid (BR)- related genes                                                                                           |
| XLOC_010301 | Cs4g04730 | −1.10          | cycloartenol synthase 1 (CAS1), involved in the biosynthesis of BRs                           |
| XLOC_012247 | Cs5g21830 | −1.12          | C-8 sterol isomerase, involved in the biosynthesis of BRs                                    |
| XLOC_002765 | −      | 2.43           | Leucine-rich receptor-like protein kinase family protein, involved in BR signaling pathways   |
| XLOC_006131 | −      | 1.52           | Leucine-rich receptor-like protein kinase family protein, involved in BR signaling pathways   |

Table 7: Summary of citrus DEGs genes involved in plant hormone metabolisms (Continued)
| ID          | Locus          | Log2Fold Change (Wt/ΔrpF) | Annotation/ Protein function                                                      |
|------------|----------------|--------------------------|----------------------------------------------------------------------------------|
| Receptor encoding genes |                |                          |                                                                                   |
| XLOC_030903 | –              | – 1.12                   | Receptor like protein 1 (RLP1), Leucine-rich-repeat-containing                      |
| XLOC_028463 | orange1.1 t01371 | – 1.36                   | Receptor like protein 1 (RLP1), Leucine-rich-repeat-containing                      |
| XLOC_022248 | Cs8g14810      | – 1.55                   | Receptor like protein 1 (RLP1), Leucine-rich-repeat-containing                      |
| XLOC_007802 | Cs3g06050      | – 1.99                   | Receptor like protein 1 (RLP1), Leucine-rich-repeat-containing                      |
| XLOC_023272 | Cs9g12160      | – 1.59                   | Receptor like protein 13 (RLP13), Leucine-rich-repeat-containing                   |
| XLOC_023274 | Cs9g12220      | – 2.09                   | Receptor like protein 13 (RLP13), Leucine-rich-repeat-containing                   |
| XLOC_023264 | Cs9g12040      | – 2.27                   | Receptor like protein 13 (RLP13), Leucine-rich-repeat-containing                   |
| XLOC_006617 | Cs3g10050      | – 2.30                   | Receptor like protein 13 (RLP13), Leucine-rich-repeat-containing                   |
| XLOC_003292 | –              | – 2.30                   | Receptor like protein 14 (RLP14), Leucine-rich-repeat-containing                   |
| XLOC_026146 | orange1.1 t02820 | – 1.15                   | Receptor like protein 15 (RLP15), Leucine-rich-repeat-containing                   |
| XLOC_028464 | orange1.1 t01372 | – 1.33                   | Receptor like protein 15 (RLP15), Leucine-rich-repeat-containing                   |
| XLOC_025415 | orange1.1 t01415 | – 1.66                   | Receptor like protein 15 (RLP15), Leucine-rich-repeat-containing                   |
| XLOC_006615 | Cs3g10010      | – 2.08                   | Receptor like protein 15 (RLP15), Leucine-rich-repeat-containing                   |
| XLOC_015519 | Cs6g12110      | – 2.13                   | Receptor like protein 15 (RLP15), Leucine-rich-repeat-containing                   |
| XLOC_023261 | Cs9g11990      | – 2.55                   | Receptor like protein 15 (RLP15), Leucine-rich-repeat-containing                   |
| XLOC_030349 | orange1.1 t05075 | – 1.31                   | Receptor like protein 22 (RLP22), Leucine-rich-repeat-containing                   |
| XLOC_013590 | Cs5g13820      | – 1.00                   | Receptor like protein 33 (RLP33), Leucine-rich-repeat-containing                   |
| XLOC_005928 | Cs2g30850      | – 1.63                   | Receptor like protein 35 (RLP35), Leucine-rich-repeat-containing                   |
| XLOC_001914 | Cs1g11900      | – 2.04                   | Receptor like protein 54 (RLP54), Leucine-rich-repeat-containing                   |
| XLOC_030467 | orange1.1 t05273 | – 1.52                   | Receptor like protein 56 (RLP56), Leucine-rich-repeat-containing                   |
| XLOC_030282 | orange1.1 t04923 | – 2.60                   | Receptor like protein 56 (RLP56), Leucine-rich-repeat-containing                   |
| XLOC_030151 | orange1.1 t06047 | – 1.12                   | Receptor like protein 6 (RLP6), Leucine-rich-repeat-containing                     |
| XLOC_006431 | Cs3g06220      | – 1.20                   | Receptor like protein 6 (RLP6), Leucine-rich-repeat-containing                     |
| XLOC_005373 | Cs2g19490      | – 1.17                   | Receptor like protein 7 (RLP7), Leucine-rich-repeat-containing                     |
| XLOC_031340 | –              | – 2.93                   | Receptor like protein 9 (RLP9), Leucine-rich-repeat-containing                     |
| XLOC_006131 | –              | – 1.52                   | Receptor-like protein kinase family protein, Leucine-rich-repeat-containing        |
| XLOC_012876 | Cs5g34310      | – 1.09                   | Receptor-like protein kinase family protein, Leucine-rich-repeat-containing        |
| XLOC_015528 | Cs6g12270      | – 2.24                   | Receptor-like protein kinase family protein, Leucine-rich-repeat-containing        |
| XLOC_002765 | –              | – 2.43                   | Receptor-like protein kinase family protein, Leucine-rich-repeat-containing        |
| XLOC_026533 | orange1.1 t03518 | – 1.22                   | Disease resistance protein (TIR-NBS-LRR class) with transmembrane receptor activity |
| XLOC_029900 | orange1.1 t04292 | – 1.30                   | Disease resistance protein (TIR-NBS-LRR class) with transmembrane receptor activity |
| XLOC_014032 | Cs5g22400      | – 1.40                   | Disease resistance protein (TIR-NBS-LRR class) with transmembrane receptor activity |
| XLOC_006408 | Cs3g05870      | – 1.14                   | Disease resistance protein (CC-NBS-LRR class) family                            |
| XLOC_006396 | Cs3g05690      | 1.99                     | Disease resistance protein (TIR-NBS-LRR class) with transmembrane receptor activity |
| XLOC_029612 | orange1.1 t03700 | 1.14                     | Disease resistance protein (TIR-NBS-LRR class) with transmembrane receptor activity |
| XLOC_006400 | Cs3g05760      | 1.09                     | Disease resistance protein (TIR-NBS-LRR class) with transmembrane receptor activity |
| XLOC_014130 | Cs5g24240      | 1.08                     | Disease resistance protein (TIR-NBS-LRR class) with transmembrane receptor activity |
| Transcription factor encoding genes |                |                          |                                                                                   |
| XLOC_013026 | Cs5g03010      | 1.08                     | WRKY transcription factor family protein (WRKY22)                                 |
| XLOC_017469 | Cs7g06330      | 1.17                     | WRKY transcription factor family protein (WRKY18)                                 |
| XLOC_016450 | Cs6g10120      | 1.19                     | WRKY transcription factor family protein (WRKY54)                                 |
| XLOC_019872 | Cs7g23080      | 1.46                     | MYB transcription factor family protein                                           |
infection by the wild type Xac compared to the ΔrpfF mutant to limit the pathogen colonization.

Discussion

The in planta DSF/Rpf-mediated QS regulon of Xac

The results indicate that the DSF deficiency altered in planta expression of 202 genes in Xac, with a remarkable downregulation of different sets of genes functionally involved in stress tolerance, nutrition uptake and metabolisms, signal transduction, transcriptional regulation, and virulence. These findings support the hypothesis that the DSF/Rpf-mediated QS in Xac modulates diverse pathogenesis traits to promote bacterial adaptation to the host environment for a successful infection (Fig. 6). For example, Xac cells have to counteract environmental stresses and plant generated- oxidative stress during infection on citrus host [48, 63]. Our results showed that DSF/Rpf-mediated QS contributes to stress tolerance of Xac by positively regulating the expression of catalase, drug resistance translocase, defense protein-degrading endoproteinase, and the MFS drug transporter (Table 1). These enzymes are collectively important for bacterial resistance against diverse stresses from the environment and/or host organisms and thus for a successful infection [38, 64, 65]. DSF/Rpf-mediated QS also positively regulates the biosynthesis of trehalose, which protects Xac cells from osmotic and oxidative stresses to enable bacterial colonization in host plants [40], i.e., in the apoplast, an osmotic stressful environment [66].

The plant apoplast is low in nitrogen and rich in plant-derived sugars such as fructose [67]. Xac has adapted to the apoplast with diverse nutrient acquisition strategies evolved, including diverse enzymes for plant cell wall degradation, amino acid metabolism, carbohydrate metabolism and transportation [63]. The findings in this study indicate that Xac exploits the DSF/Rpf-mediated QS to regulate nutrition utilization during host infection (Table 2; Table 3). Interestingly, the DSF/Rpf-mediated QS positively regulates the expression of phosphate transporter encoding genes, the homologues of which in X. axonopodis pv. glycines, the causal agent of bacterial pustule of soybean, have been demonstrated to be strongly expressed at early stages of infection and required for bacterial growth in host plants to promote disease [68]. DSF/Rpf-mediated QS also regulates ferric iron uptake of Xac in planta (Table 3). It has been reported that Xanthoferrin, a α-hydroxycarboxylylate-type siderophore produced by Xcc is required for its optimum virulence [69]; and, DSF positively regulates the functions involved in ferric iron uptake to promote in planta growth of X. oryzae pv. oryzicola [70]. However, there is no evidence that iron is limited or available to Xac cells grown in planta. The functional role of DSF/Rpf-regulated ferric iron uptake in Xac biology and pathogenesis remains to be determined.

Importantly, the DSF/Rpf-mediated QS differentially regulated the expression of 12 determined or putative signal transducers and/or transcriptional regulators, most of which were downregulated, including the NtrB/C two-component system (Table 4). The NtrB/C system interacts with the RpfC/G system in responding to DSF signal to regulate sigma54-dependent promoters in Xac in vitro [44]. Our findings suggested that the DSF signal negatively regulates sigma54-dependent promoters through the RpfCG·NtrBC-sigma54 pathway in Xac during early stages of host infection. The functional roles of the other signal transducers and/or transcriptional regulators regulated
by the DSF/Rpf-mediated QS remain unknown. Collectively, the results suggested that DSF-mediated signaling might be linked with diverse regulators to enable complex patterns of gene expression to be employed by Xac to favor infection in host plants, which deserves further investigations.

Comparison of the in planta and in vitro DSF/Rpf regulons revealed that a set of 31 genes were commonly differentially regulated by DSF/Rpf under the two environment conditions. There are a large number of unique genes in the in planta regulon that were not regulated by DSF/Rpf in vitro (Additional file 5: Table S5). A couple of reasons could explain the differences among the in planta and in vitro DSF/Rpf regulons. It could be because of the difference in cell density of Xac in the two experimental conditions: approximately $10^8$ CFU/cm$^2$ of leaf tissues for in planta experiments (Fig. 1b) and $10^9$ to $10^{10}$ CFU/ml of growth medium for in vitro experiments [17], as the QS regulates expression of genes in a cell density-dependent manner. It also could be because that the DSF/Rpf – mediated QS might play diverse roles in regulating gene expression of Xac under different environment conditions. Several subsets of unique genes within the in planta regulon that were downregulated are involved in cell surface adhesion, stress tolerance (catalase and trehalose), signal transduction, transcription, and virulence factors, which collectively promote bacterial adaptation to the host environment to favor infection. The transcriptional alterations of citrus in response to DSF-mediated X. citri subsp. citri infection are characterized by the downregulation of photosynthesis, plant immune receptor-like proteins or receptor-like kinases including NBS-LRRs, NB-ARC resistance proteins, MYB and WRKY transcription factors, and pathogenesis-related (PR) proteins. Changes of phytohormone metabolism and signaling were also triggered by DSF-mediated X. citri subsp. citri infection, probably leading to increased accumulation of auxin, ethylene and jasmonic acid (JA), and decreased accumulation of brassinosteroid (BR), cytokinin and salicylic acid (SA), which may benefit the pathogen. Solid arrows with plus symbols indicate positive regulation and dashed arrows with minus symbols indicate negative regulation. Solid lines indicate information flow.

Fig. 6 Hypothetical model of the modulation of citrus - Xanthomonas citri subsp. citri interactions by the DSF/Rpf- mediated quorum sensing (QS) during early stages of infection. Representative proteins and metabolic processes with important roles in plant-pathogen interactions are shown. Plant and bacterial molecules are depicted in light blue and red, respectively. DSF/Rpf- mediated QS modulates expression of diverse bacterial traits including adhesion, nutrition acquisition, stress tolerance (catalase and trehalose), signal transduction, transcription, and virulence factors, which collectively promote bacterial adaptation to the host environment to favor infection. The transcriptional alterations of citrus in response to DSF-mediated X. citri subsp. citri infection are characterized by the downregulation of photosynthesis, plant immune receptor-like proteins or receptor-like kinases including NBS-LRRs, NB-ARC resistance proteins, MYB and WRKY transcription factors, and pathogenesis-related (PR) proteins. Changes of phytohormone metabolism and signaling were also triggered by DSF-mediated X. citri subsp. citri infection, probably leading to increased accumulation of auxin, ethylene and jasmonic acid (JA), and decreased accumulation of brassinosteroid (BR), cytokinin and salicylic acid (SA), which may benefit the pathogen. Solid arrows with plus symbols indicate positive regulation and dashed arrows with minus symbols indicate negative regulation. Solid lines indicate information flow.

Citrus transcriptional responses to DSF/Rpf-mediated Xac infection

Gene expression data indicated that significant transcriptional alterations occurred in citrus plants in
response to DSF/Rpf-mediated Xac infection, which caused various changes in plant immunity and physiology, thus favoring the pathogen infection. Especially, a large group of genes differentially expressed, related to plant hormone biosynthesis, transportation, metabolism, and associated signal transduction (Table 7). The results suggested the existence of elevated levels of auxin in wild type Xac infected leaves compared with the ΔrpfF mutant infected leaves. Auxin has been shown to promote citrus canker development [71]; and auxin pathways play a role in tomato bacterial wilt caused by Ralstonia solanacearum [72]. Therefore, it is likely that the alterations in expression of auxin biosynthesis, mobilization and signaling genes in response to the DSF/Rpf-mediated Xac infection are associated with the citrus canker disease development. Additionally, cytokinin biosynthesis genes were downregulated and cytokinin metabolic genes were upregulated, implying decreased accumulation of cytokinin in wild type Xac infected leaves. Cytokinin has been shown to regulate plant defense responses in a dosage-dependent manner: strong activation of cytokinin signaling confers resistance to biotrophic pathogens via increased SA accumulation; by contrast, weak activation of cytokinin signaling suppresses pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) [73]. Our results suggested that the DSF/Rpf-mediated Xac infection modulates cytokinin accumulation and thus avoids strong activation of cytokinin signaling to promote host susceptibility. Another interesting finding is the upregulation of genes involved in the biosynthesis of and response to ethylene in wild type Xac infected leaves. Xac infection activates ethylene biosynthesis and signaling in citrus plants [61]. Ethylene is usually involved in plant defense responses against necrotrrophic pathogens [74], thus it is possible that the successful establishment of Xac infection is favored by the development of inadequate plant defenses.

Notably, genes for JA biosynthesis and for SA production (i.e., the MeSA esterase) were upregulated in wild type Xac infected leaves, while genes for SA metabolic process and for BR biosynthesis or responses were downregulated (Table 7). Earlier reports showed that certain antagonistic relationships occur between BR and JA, JA and SA pathways, and BR signaling negatively regulates plant defense against pathogens [55, 75, 76]. Both biotropic and hemibiotrophic pathogens employ the antagonism between JA and SA pathways and activate JA signaling to promote infection [77, 78]. The findings in this study implied that the DSF/Rpf-mediated Xac infection may activate the JA signaling pathway and repress BR signaling to benefit the pathogen during early stages of infection.

Gene expression levels point to that the activity of DSF/Rpf-mediated QS might induce plant basal defenses and repress secondary defenses of citrus to promote Xac infection (Table 8; Additional file 6: Table S6; Additional file 7: Table S7; Additional file 8: Table S8). Remarkably, many plant immune receptor-like proteins or receptor-like kinases proteins were downregulated by DSF/Rpf-mediated Xac infection (Table 8), which are believed to perceive extracellular molecules, including microbe/pathogen-associated molecular patterns (M/PAMP) and environmental stimuli to induce plant basal resistance [79, 80]. In addition, four putative NB-LRR family proteins were also downregulated by DSF/Rpf-mediated Xac infection (70 downregulated versus 42 upregulated) (Additional file 6: Table S6), especially in the group of immune receptors (34 downregulated versus 4 upregulated) (Table 8).

It is not clear how the activity of DSF/Rpf-mediated QS triggers plant basal defenses and represses secondary defenses of citrus plants. One possible explanation might lie in the observations that the DSF signal molecule itself could elicit plant defense response in Xanthomonas–host plant interactions and wild-type Xanthomonas spp. can suppress the DSF-induced defense responses by the production of the EPS xanthan and T3SS effectors [18]. Our results showed that the DSF/Rpf-mediated QS did not regulate or affect the production of the EPS xanthan by Xac in citrus during early stages of infection, but negatively regulated the expression of a putative T3SS effector (XAC3085) (Table 5). The homologue of XAC3085 in X. campestris pv. vesicatoria (also termed X. euvesicatoria), the causal agent of bacterial spot disease on pepper and tomato, was determined as a T3SS effector, named XopK, whose function remains unknown but seems not to contribute to the virulence of the pathogen [82]. Xac might suppress the DSF molecule elicited plant defense responses through the EPS and/or the T3SS effectors that are not affected by the DSF/Rpf-mediated QS during host infection. Another possible reason might be the functional interplay between the bacterial T2SS and T3SS in modulating plant defense responses and promoting disease as observed in the X. oryzae pv. oryzae – rice interactions, where the bacterial T2SS secreted virulence factors: the ClsA cellulase and CbsA cellobiosidase, induced innate rice defense responses that were suppressed by T3S effectors [83]. We found that the DSF/Rpf-mediated QS positively regulates the expression of the homologue (engXCA/XAC0612) of the ClsA cellulase (Table 2). Therefore,
wild-type Xac may suppress, in a T3SS-dependent manner, the citrus plant defense responses probably induced by the T2SS effector cellulase (engXCA/XAC0612) to enable successful infection.

Conclusions

In conclusion, this work provides an in-depth transcriptomic analysis of DSF/Rpf-mediated QS regulation from both pathogen and host sides during the biotrophic interactions between Xac and citrus. Based on the results obtained, a model was presented that describes the major molecular and physiological aspects regulated by the DSF/Rpf-mediated QS during early stages of infection (Fig. 6). The findings support the hypothesis that the DSF/Rpf-mediated QS in Xac modulates diverse pathogenesis traits to promote bacterial adaptation to the host environment, and triggers various changes in plant immunity and physiology favoring the pathogen for successful infection. Taken together, the present work has provided novel insights into the role of the DSF/Rpf-mediated QS regulatory system in the pathogenic interactions between Xanthomonas and its host plants and expanded our current knowledge of DSF-mediated QS regulation, and adds to our general understanding of plant-pathogen interactions.

Additional Files

Additional file 1: Table S1. Primers used for qRT-PCR assays for experimental validation (DOCX 18 kb)
Additional file 2: Table S2. Summary of the RNA-seq data (DOXC 13 kb)
Additional file 3: Table S3. Detail of the DEGs of Xanthomonas citri subsp. citri regulated by DSF/Rpf –mediated QS (DOXC 35 kb)
Additional file 4: Table S4. Detail of the DEGs of citrus in response to DSF/Rpf-mediated QS (DOCX 183 kb)
Additional file 5: Table S5. Comparison of the in vitro and in planta DSF/Rpf-mediated QS regulons of Xanthomonas citri subsp. citri (XLXS 29 kb)
Additional file 6: Table S6. Differentially expressed citrus genes related to plant defense responses (XLXS 17 kb)
Additional file 7: Table S7. Differentially expressed citrus genes involved in secondary metabolisms (XLXS 11 kb)
Additional file 8: Table S8. Differentially expressed citrus genes involved in cell wall modifications (XLXS 13 kb)

Abbreviations

bp: Base pair; cDNA: Complementary DNA; COG: Clusters of Orthologous Groups; DEGs: Differentially expressed genes; DNA: Deoxyribonucleic acid; DSF: Diffusible signal factor; EPS: Extracellular polysaccharides; FDR: False rate discovery; FPKM: Fragments per kilobase of exon per million mapped reads; kb: Kilobases; log2FC: Log of fold change in base 2; LPS: Lipopolysaccharides; min: Minute; mIM: Millimolar; miRNA: Messenger RNA; PCR: Polymerase chain reaction; pH: Hydrogenic potential; qRT-PCR: Quantitative reverse transcription PCR; QS: Quorum sensing; RNA: Ribonucleic acid; RNA-Seq: RNA sequencing; Rpf: Regulation of pathogenicity factors; rpm: Rotations per minute; T2SS: Type II secretion system; T3SS: Type III secretion system; Xac: X. citri subsp. citri; Xcc: X. campestris pv. campestris; μg: Micrograms; μM: Micromolar

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Availability of data and materials

The RNA sequence dataset supporting the results in this article is available from the NCBI under the bioproject no. PRJNA421992 with the SRA accession no. SRP126698 (https://www.ncbi.nlm.nih.gov/sra/?term=SRP126698).

Author’s contributions

NW, JL, and LL conceived and designed the experiments. LL and JL performed the experiments. LL, JL, YZ, and NW analyzed data. LL, JL, YZ, and NW wrote the manuscript. All authors read and approved the manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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