RpfC regulates the expression of the key regulator hrpX of the hrp/T3SS system in Xanthomonas campestris pv. campestris

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Abstract

Background: The Gram-negative phytopathogenic bacterium Xanthomonas campestris pv. campestris recruits the hrp/T3SS system to inject pathogenicity effector proteins into host cells and uses the rpf/DSF cell-cell signaling system to regulate the expression of virulence factors such as extracellular enzymes and polysaccharide. Whether these two systems have any connection is unknown.

Methods: Positive regulator candidates affecting hrpX expression were identified by sacB strategy. The transcriptional expression was determined by qRT-PCR and GUS activity analysis. Transcriptome analysis was performed by RNA deep-sequencing. The hypersensitive response (HR) was determined in the nonhost plant pepper ECW-10R and electrolyte leakage assay.

Results: Mutation of the gene encoding the sensor RpfC of the rpf/DSF system significantly reduced the expression of hrpX, the key regulator of the hrp/T3SS system, all of the genes in the hrp cluster and most reported type III effector genes. Mutation of rpfG did not affect the expression of hrpX. The rpfC mutant showed a delayed and weakened HR induction.

Conclusions: RpfC positively regulates the expression of hrpX independent of RpfG, showing a complex regulatory network linking the rpf/DSF and hrp/T3SS systems.

Keywords: Xanthomonas, RpfC, hrpX

Background

The Gram-negative bacterium Xanthomonas campestris pathovar campestris (Xcc) is the causal agent of black rot disease, one of the most destructive diseases of cruciferous crops worldwide [1]. This pathogen can infect almost all members of the crucifer family (Brassicaceae), including many important vegetables, the major oil crop rape, and the model plant Arabidopsis thaliana. Over the past several decades, Xcc has been used as a model bacterium for studying molecular mechanisms of bacterial pathogenicity [2]. The entire genome sequences of a number of strains such as ATCC33913, 8004, and B100 have been determined [3–5] and a large number of genes associated with essential virulence have been identified. Among them, rpf (regulation of pathogenicity factors) and hrp (hypersensitive response and pathogenicity) clusters of genes are essential for pathogenicity of Xcc [6–8].

The Xcc rpf cluster of genes consists of at least nine genes (rpfA to rpfI). This gene cluster is involved in the quorum sensing system, controlling the synthesis of a diffusible signal factor (DSF) and regulating extracellular plant cell wall-degrading enzymes and extracellular polysaccharide (EPS) production as well as biofilm formation [6, 9–11]. The role of rpfC, rpfF and rpfG genes has been extensively studied [9–17]. The rpfF gene encodes an enzyme responsible for synthesizing the DSF molecules, which are secreted into extracellular environment [16]. The proteins encoded by rpfC and rpfG compose a two-component signal transduction system which is implicated in DSF perception and signal transduction [9, 12, 13]. RpfC acts as the histidine kinase sensor in the two component regulatory system to sense the environmental DSF signal, leading to

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activation of RpfG as a cyclic di-GMP phosphodiesterase. The activation of RpfG then leads to a reduction of cyclic di-GMP level which promotes synthesis of extracellular enzymes and EPS [9, 12, 13]. In addition, it is known that cyclic di-GMP effects on the synthesis of extracellular enzymes and EPS involve the transcriptional activator Clp (cAMP receptor-like protein). Cyclic di-GMP binds to Clp, thus preventing binding of Clp to the promoters of target genes that include those encoding extracellular enzymes and EPS biosynthesis [13–17].

In addition to the rpf/DSF regulatory system, the pathogenicity of Xcc is also dependent on the hrp cluster of genes. The hrp genes are associated with pathogen-induced hypersensitive response (HR), a disease-resistant phenomenon on the infection sites of resistant hosts and nonhost plants, and pathogen's pathogenicity in susceptible hosts. Most hrp genes in the cluster encode the type III secretion system (T3SS) that translocates effector proteins into host cells and is highly conserved among Gram-negative pathogenic bacteria [18–20]. In Xcc, the hrp cluster is composed of six main operons (hrpA to hrpF) which harbor more than 20 different genes [7]. The expression of the operons is regulated by the AraC-type transcriptional activator HrpX [21]. The expression of hrpX is positively regulated by a two-component signal transduction system composed of HpaS and HrpG [21, 22]. HpaS is a histidine kinase sensor and HrpG is an OmpR family response regulator [22]. It is clear that the expression of the hrp genes including the regulators hrpG and hrpX is expressed at low levels in nutrient rich media but induced in plant tissues or in certain minimal media [7, 21].

As the hrp genes are induced in minimal media but expressed at low levels in nutrient rich media, the studies on the hrp/T3SS system were commonly carried out in certain minimal media. On the contrary, the rpf/DSF system is studied in nutrient rich media. To our knowledge, no work on the link between rpf/DSF and hrp/T3SS systems has been reported. The aim of this work was to identify upstream regulators of hrpX in Xcc. We employed the sacB strategy [23] to screen mutations that affect the expression of hrpX. Interestingly, we found that a mutation in the rpfC gene of the rpf/DSF system significantly reduced the expression of hrpX. Here, we provide evidences showing that RpfC positively regulates hrpX.

**Methods**

**Bacterial strains, plasmids and growth conditions**

The bacterial strains and plasmids used in this work are listed in Table 1. Xcc strains were grown at 28 °C in nutrient rich medium NYG [24] or minimal media MMX (23.8 mM glucose, 3.87 mM sodium citrate, 15.1 mM (NH4)2SO4, 0.81 mM MgSO4, 23 mM K2HPO4, 44 mM KH2PO4, pH 7.0) [24] and XCM1 (20 mM succinic acid, 0.15 g/l casamino acids, 7.57 mM (NH4)2SO4, 1 mM MgSO4, 60.34 mM K2HPO4, 33.07 mM KH2PO4, pH 6.6) [25]. Antibiotics were used at the following final concentrations as required: ampicillin (Amp), 100 μg/ml; gentamycin (Gm), 10 μg/ml; kanamycin (Kan), 25 μg/ml; rifampicin (Rif), 50 μg/ml; and tetracycline (Tc), 15 μg/ml for *Escherichia coli* and 5 μg/ml for Xcc. *E. coli* strains were grown in Luria-Bertani medium (LB, per liter: tryptone 10 g, yeast extract 5 g, NaCl 10 g) at 37 °C. The triparental conjugation between Xcc and *E. coli* strains was performed as described by Daniels and associates [24]. Restriction enzymes and DNA ligase were used in accordance with the manufacturer's instructions (Promega, Madison, Wisconsin, USA).

**Screen for mutations affecting the expression of hrpX**

In order to screen the genes influencing the expression of hrpX, the sacB system [26] was employed. The 1419-bp sacB gene without the start codon ATG was amplified from the plasmid pK18mobsacB [27] (Table 1) using the primer pair sacB-F/sacB-R (Table 2). After confirmation by sequencing, the amplified sacB gene was ligated into the plasmid pLAFR6 [28] (Table 1), yielding the recombinant plasmid pL6sacB (Table 1). The promoter of hrpX was then in-frame cloned into pL6sacB, generating the plasmid pL6hrpXsacB, in which the sacB gene is driven by the hrpX promoter (Table 1). The plasmid pL6hrpXsacB was introduced into Xcc wild type strain 8004 from *E. coli* by triparental conjugation, yielding the strain 8004/pL6hrpXsacB (Table 1). The bacterial cells of strain 8004/pL6hrpXsacB were treated to be competent status and mutated by the EZ-Tn5™ transposon using a commercial EZ-Tn5™ transposon kit (Epichent Biotechnology), followed by selecting mutant colonies on the plates of MMX minimal medium containing Rif, Kan, Tc and 5% sucrose.

To map the transposon insertion sites in the obtained mutants, the total DNA of each mutant was isolated and digested with *Eco*RI (no *Eco*RI site within the transposon), and then cloned into the plasmid pUC19 [29] (Table 1). The resulting recombinant plasmid was transformed into *E. coli* strain JM109 [29] (Table 1) and transformants were selected by Kan (for the transposon) plus Amp resistance. The recombinant plasmid was isolated from the obtained Kan- and Amp-resistant transformants and the DNA sequences flanking the transposon were identified by sequencing the recombinant plasmid using the primers KAN-2 FP-1 or KAN-2 RP-1 (Table 2).

**Construction of mutants and GUS reporters**

An rpfC deletion mutant was generated by the methods described previously [30]. Briefly, two DNA fragments flanking rpfC gene were generated by PCR using the primer pairs RpfC-1-FOR/RpfC-1-REV and RpfC-2-FOR/RpfC-2-REV (Table 2). The resultant DNA fragments were cleaved with *Bam*HI and ligated. The fusion fragments were then amplified using the ligation mixture as
the template and the primer pair RpfC-1-FOR/RpfC-2-REV and cloned into the Snal site of vector pK18mobsacB and transformed into E. coli strain JM109. After sequence verification, the obtained recombinant plasmid was mobilized into Xcc strain 8004 by triparental conjugation. Transconjugants were firstly selected on NYG medium supplemented with Rif and Kan. The second selection was made on NYG medium containing 5% sucrose and Rif for resolution of the vector by a second crossover event. The in-frame deletion of rpfC was confirmed by PCR and sequencing.

To construct Xcc hrpG and hrpX promoter-gusA transcriptional fusion reporters, the promoter regions of hrpG and hrpX were amplified from Xcc strain 8004 using the primer sets PhrpG-F/PhrpG-R and PhrpX-F/PhrpX-R (Table 2), respectively. The amplified hrpG promoter fragment and hrpX promoter fragment were double digested with SacI plus XbaI and EcoRI plus KpnI, respectively, then ligated into the plasmid pUC19 (Table 1). The resulting recombinant plasmids were then transformed into E. coli JM109. Transformants were selected on LB medium...

| Strains or plasmids | Relevant characteristics | Source |
|---------------------|--------------------------|--------|
| X. c. pv. campestris | Wild type; Rif<sup>r</sup> | [24]   |
| XB001               | 8004/plG6xpXsacB with a Tn5 insertion in Xc_4007; Rif<sup>r</sup>; Kan<sup>r</sup>; Tc<sup>r</sup> | This work |
| XB002               | 8004/plG6xpXsacB with a Tn5 insertion in the intergenic region between the ORFs Xc_1510 and Xc_1511; Rif<sup>r</sup>; Kan<sup>r</sup>; Tc<sup>r</sup> | This work |
| XB003               | 8004/plG6xpXsacB with a Tn5 insertion in Xc_2333; Rif<sup>r</sup>; Kan<sup>r</sup>; Tc<sup>r</sup> | This work |
| XB004               | 8004/plG6xpXsacB with a Tn5 insertion in Xc_1192; Rif<sup>r</sup>; Kan<sup>r</sup>; Tc<sup>r</sup> | This work |
| XB005               | 8004/plG6xpXsacB with a Tn5 insertion in Xc_3951; Rif<sup>r</sup>; Kan<sup>r</sup>; Tc<sup>r</sup> | This work |
| XB006               | 8004/plG6xpXsacB with a Tn5 insertion in Xc_0124; Rif<sup>r</sup>; Kan<sup>r</sup>; Tc<sup>r</sup> | This work |
| 8004/plG6xpXsacB    | 8004 harboring plasmid plG6xpXsacB; Rif<sup>r</sup>; Tc<sup>r</sup> | This work |
| ΔrpfC               | rpfC in frame deletion mutant of 8004; Rif<sup>r</sup> | This work |
| CΔrpfC              | ΔrpfC harboring plasmid pLCpfc; Rif<sup>r</sup>; Tc<sup>r</sup> | This work |
| ΔrpfG               | rpfG in frame deletion mutant of 8004; Rif<sup>r</sup> | [17]   |
| ΔavrBs1             | avrBs1 in frame deletion mutant of 8004; Rif<sup>r</sup>; Gm<sup>r</sup> | [44]   |
| 8004/pGUShrpG       | 8004 harboring plasmid pGUShrpG; Rif<sup>r</sup>; Tc<sup>r</sup> | This work |
| ΔrpfC/pGUShrpG      | ΔrpfC harboring plasmid pGUShrpG; Rif<sup>r</sup>; Tc<sup>r</sup> | This work |
| 8004/pGUShrpX       | 8004 harboring plasmid pGUShrpX; Rif<sup>r</sup>; Tc<sup>r</sup> | This work |
| ΔrpfC/pGUShrpX      | ΔrpfC harboring plasmid pGUShrpX; Rif<sup>r</sup>; Tc<sup>r</sup> | This work |
| E. coli              | RecA1, endA1, gyrA96, thi, supE44, relA1 Δ(lac-proAB)/F<sup>′</sup>[traD36, lacI q, lacZ ΔM15] | [29]   |

| Plasmids             | Relevant characteristics | Source |
|----------------------|--------------------------|--------|
| pUC19                | Cloning vector; Amp<sup>r</sup> | [28]   |
| pLAFR6               | Broad host range IncP cloning cosmid; Tc<sup>r</sup> | [28]   |
| pK18mobsacB          | Suicide plasmid in Xcc; Mob<sup>+</sup> Tra<sup>+</sup>; Kan<sup>r</sup> | [27]   |
| pLGUS                | pLAFR6 containing a 1832-bp gusA ORF (excluding ATG), Tc<sup>r</sup> | [31]   |
| pLSacB               | pLAFR6 containing a 1419-bp sacB gene, Tc<sup>r</sup> | This work |
| pK18mobsacB          | pK18mobsacB containing the two flanking fragments of rpfC; Kan<sup>r</sup> | This work |
| pUCP/hrpG            | pUC19 containing hrpG promoter; Amp<sup>r</sup> | This work |
| pUCP/hrpX            | pUC19 containing hrpX promoter; Amp<sup>r</sup> | This work |
| pGUShrpG             | pLAFR6 containing hrpG promoter in frame fused with gus gene; Tc<sup>r</sup> | This work |
| pGUShrpX             | pLAFR6 containing hrpX promoter in frame fused with gus gene; Tc<sup>r</sup> | This work |
| pLSacB               | pLAFR6 containing hrpX promoter in frame fused with sacB gene; Tc<sup>r</sup> | This work |
| pLCpfc               | pLAFR6 containing the sequenced whole ORF of rpfC; Tc<sup>r</sup> | This work |

<sup>Amp<sup>r</sup>, ampicillin-resistant; Gm<sup>r</sup>, gentamicin-resistant; Kan<sup>r</sup>, kanamycin-resistant; Rif<sup>r</sup>, rifampicin-resistant; Tc<sup>r</sup>, tetracycline-resistant</sup>
| Primer name | Primer sequence | Product length (bp) |
|-------------|-----------------|--------------------|
| sacB-F      | CCCTCTAGA ATCAAAAAGTTGCAAAACAAG | 1419 |
| sacB-R      | CCCGTCGAC AAATAAAAAGAAAAATGCAATTAG | |
| RpfC-1-F    | ATTTGCGCTGATCTGGTCTAC | 553 |
| RpfC-1-R    | CGGATCC AGACTTCATAGACGCGCTAGG | |
| RpfC-2-F    | ACAGCGACGTGTTCAATCTGGGCG | 665 |
| RpfC-2-R    | GGGGAGCTC GGTGTTCGGCACGCAGATGCGC | |
| PhrpG-F     | GGAGAGCTC GGTGTTCGCCAGCAGATGC | 104 |
| PhrpG-R     | GGATTTGCGCTGATCTGGTCTAC | |
| PhrpX-F     | GGATTTGCGCTGATCTGGTCTAC | |
| PhrpX-R     | GGATTTGCGCTGATCTGGTCTAC | |
| KAN-2 FP-1  | ACCTACACAAACAGCTTCATCAACC | |
| KAN-2 RP-1  | GCAATGTAACATCAGAGATTTTGAG | |
| XC0052F     | ACAGATTGGTCTCGCAGGTC | 104 |
| XC0052R     | GGCAATGCTCTGATCGGTCT | |
| XC0241F     | AGCCGCATTCAGCCGCAACGGA | 92 |
| XC0241R     | ACCACGGCGCTGCTGGTGTTCAA | |
| XC1553F     | TTTTCCGCGTCTGGTGTTCAA | |
| XC1553R     | TTTTCCGCGTCTGGTGTTCAA | |
| XC0204F     | AGCAATGTAACATCAGAGATTTTGAG | |
| XC0204R     | GCAATGTAACATCAGAGATTTTGAG | |
| XC0201F     | AGCAATGTAACATCAGAGATTTTGAG | |
| XC0201R     | GCAATGTAACATCAGAGATTTTGAG | |
| XC0208F     | AGCAATGTAACATCAGAGATTTTGAG | |
| XC0208R     | GCAATGTAACATCAGAGATTTTGAG | |
| XC0201F     | AGCAATGTAACATCAGAGATTTTGAG | |
| XC0201R     | GCAATGTAACATCAGAGATTTTGAG | |
| XC0208F     | AGCAATGTAACATCAGAGATTTTGAG | |
| XC0208R     | GCAATGTAACATCAGAGATTTTGAG | |
| XC0201F     | AGCAATGTAACATCAGAGATTTTGAG | |
| XC0201R     | GCAATGTAACATCAGAGATTTTGAG | |
| XC0208F     | AGCAATGTAACATCAGAGATTTTGAG | |
| XC0208R     | GCAATGTAACATCAGAGATTTTGAG | |
| XC0201F     | AGCAATGTAACATCAGAGATTTTGAG | |
| XC0201R     | GCAATGTAACATCAGAGATTTTGAG | |
| XC0208F     | AGCAATGTAACATCAGAGATTTTGAG | |
| XC0208R     | GCAATGTAACATCAGAGATTTTGAG | |
| XC0201F     | AGCAATGTAACATCAGAGATTTTGAG | |
| XC0201R     | GCAATGTAACATCAGAGATTTTGAG | |
| XC0208F     | AGCAATGTAACATCAGAGATTTTGAG | |
| XC0208R     | GCAATGTAACATCAGAGATTTTGAG | |
| XC0201F     | AGCAATGTAACATCAGAGATTTTGAG | |
| XC0201R     | GCAATGTAACATCAGAGATTTTGAG | |
| XC0208F     | AGCAATGTAACATCAGAGATTTTGAG | |
| XC0208R     | GCAATGTAACATCAGAGATTTTGAG | |
| XC0201F     | AGCAATGTAACATCAGAGATTTTGAG | |
| XC0201R     | GCAATGTAACATCAGAGATTTTGAG | |
| XC0208F     | AGCAATGTAACATCAGAGATTTTGAG | |
| XC0208R     | GCAATGTAACATCAGAGATTTTGAG | |
| XC0201F     | AGCAATGTAACATCAGAGATTTTGAG | |
| XC0201R     | GCAATGTAACATCAGAGATTTTGAG | |
| XC0208F     | AGCAATGTAACATCAGAGATTTTGAG | |
| XC0208R     | GCAATGTAACATCAGAGATTTTGAG | |
| XC0201F     | AGCAATGTAACATCAGAGATTTTGAG | |
| XC0201R     | GCAATGTAACATCAGAGATTTTGAG | |
| XC0208F     | AGCAATGTAACATCAGAGATTTTGAG | |
| XC0208R     | GCAATGTAACATCAGAGATTTTGAG | |
| XC0201F     | AGCAATGTAACATCAGAGATTTTGAG | |
| XC0201R     | GCAATGTAACATCAGAGATTTTGAG | |
| XC0208F     | AGCAATGTAACATCAGAGATTTTGAG | |
| XC0208R     | GCAATGTAACATCAGAGATTTTGAG | |
| XC0201F     | AGCAATGTAACATCAGAGATTTTGAG | |
| XC0201R     | GCAATGTAACATCAGAGATTTTGAG | |
| XC0208F     | AGCAATGTAACATCAGAGATTTTGAG | |
| XC0208R     | GCAATGTAACATCAGAGATTTTGAG | |
| XC0201F     | AGCAATGTAACATCAGAGATTTTGAG | |
| XC0201R     | GCAATGTAACATCAGAGATTTTGAG | |
| XC0208F     | AGCAATGTAACATCAGAGATTTTGAG | |
| XC0208R     | GCAATGTAACATCAGAGATTTTGAG | |
| XC0201F     | AGCAATGTAACATCAGAGATTTTGAG | |
| XC0201R     | GCAATGTAACATCAGAGATTTTGAG | |
| XC0208F     | AGCAATGTAACATCAGAGATTTTGAG | |
| XC0208R     | GCAATGTAACATCAGAGATTTTGAG | |
| XC0201F     | AGCAATGTAACATCAGAGATTTTGAG | |
| XC0201R     | GCAATGTAACATCAGAGATTTTGAG | |
| XC0208F     | AGCAATGTAACATCAGAGATTTTGAG | |
| XC0208R     | GCAATGTAACATCAGAGATTTTGAG | |
supplemented with IPTG, X-gal (5-Bromo-4-chloro-3-indolyl-β-D-galactoside) and Kan. The positive colonies were confirmed by PCR and sequencing, generating the plasmids pUCP
/hrpG and pUCP
/hrpX (Table 1). The promoter regions of
/hrpG and
/hrpX were excised from plasmids pUCP
/hrpG and pUCP
/hrpX and cloned into pLGUS [31] (Table 1) and transformed into E. coli JM109. Transformants were selected on LB medium supplemented with Tc. Recombinant plasmids were isolated from the obtained transformants and confirmed by PCR and restriction enzyme digestion. The confirmed recombinant plasmids were named pGUS
/hrpG and pGUS
/hrpX, respectively. These reporter plasmids were subsequently transferred into Xcc strains ΔrpfC and 8004 by triparental conjugation. Transconjugants were selected on NYG medium supplemented with Rif and Tc. The resulting transconjugants 8004/pGUS
/hrpG, ΔrpfC/pGUS
/hrpG, 8004/pGUS
/hrpX, and ΔrpfC/pGUS
/hrpX (Table 1) were further confirmed by PCR and restriction enzyme digestion.

**HR test and electrolyte leakage assay**

HR test was performed as described previously [32]. The Xcc nonhost plant pepper ECW-10R (*Capsicum annuum* cv. ECW-10R) was used. Pepper seedlings were grown in a greenhouse with 12 h day and night cycle illumination by fluorescent lamps at temperatures of 25 to 28 °C. Bacterial cells of Xcc strains from overnight cultures were washed and diluted to a concentration at an optical density at 0.01 (600 nm)
homogenized. Then, 30 μg X-100 and grinded with mortar and pestle until OD600 = 0.01 in sterile water and introduced into host

sions of Xcc substrate as described previously [34]. Bacterial suspen-

sions were diluted to a concentration of OD600 = 0.01 in 10 mM sodium phosphate buffer and measurements were carried out exactly as described previously [33]. Essentially, for each sample, four leaf disks were removed with a 0.7-cm diameter cork borer, submerged in 10 ml of distilled water, and vacuum-infiltrated. Then, the net leakage after 1 h was measured with a conductivity meter (DDS-307A). Three samples were taken for each measurement in each experiment; the experiments were repeated at least twice.

For electrolyte leakage assay, bacterial suspensions were diluted to a concentration of OD600 = 0.01 in 10 mM sodium phosphate buffer and measurements were carried out exactly as described previously [33]. Essentially, for each sample, four leaf disks were removed with a 0.7-cm diameter cork borer, submerged in 10 ml of distilled water, and vacuum-infiltrated. Then, the net leakage after 1 h was measured with a conductivity meter (DDS-307A). Three samples were taken for each measurement in each experiment; the experiments were repeated at least twice.

**GUS activity assay**

Xcc cells from overnight culture in NYG medium were resuspended in XCM1 medium to a final optical density of 0.1 (600 nm) and incubated for 24 h. Then, 1 ml of the culture was transferred to another 10 ml fresh XCM1 medium and incubated for 24 h. To determine the β-glucuronidase (GUS) activity of the bacterial cells, 200 μl cultures for each strain were mixed with 40 μl methylbenzene and vortexed. The supernatant was then taken for GUS activity assay. The GUS activity assay was performed by measurement of the OD415 using ρ-nitrophenyl-β-D-glucuronide as substrate as described previously [34].

**Histochemical GUS staining**

Chinese radish cv. Manshenhong seedlings with four fully expanded leaves were used for inoculation. Histochemical GUS staining was performed by using 5-bromo-4-chloro-3-indolylglucuronide (Promega) as a substrate as described previously [34]. Bacterial suspensions of Xcc strains were diluted to a concentration of OD600 = 0.01 in sterile water and introduced into host plant leaves. For GUS activity quantification of bacterial cells in the plant leaves, the fluorogenic substrate 4-methylumbelliferyl-β-D-glucuronide was used following the method described previously [35]. For plant protein extraction, 10 mg plant leaves were added to 1 ml of cold GUS extraction buffer [50 mM Na2PO4, pH 7.0, 10 mM β-mercaptoethanol, 10 mM EDTA, 0.1% (w/v) sodium lauryl sarcosine, and 0.1% (w/v) Triton X-100] and grinded with mortar and pestle until homogenized. Then, 30 μl 0.1% SDS and 60 μl chloroform were added. After 10 s vortexes, samples were transformed into micro-centrifuge tubes and centrifuged for 8 min at 8000 rcf. The plant extract protein was quantified and immediately tested by adding the GUS assay buffer [2 mM 4-MUG (4-Methyl-1-umbelliferyl-β-D-Glucuronide)]. The assay was performed using 5-bromo-4-chloro-3-indolylglucuronide (X-Gluc) (Promega) as substrate, essentially as described previously [35]. At least four wells for each concentration of MUG (two with plant extract and two with extraction buffer to serve as blanks and correct for any nonenzymatic hydrolysis of MUG). Final MUG concentrations of 10 μM, 30 μM, 50 μM, 70 μM, and 90 μM were used for plotting a standard curve. A 30 μM MUG was chosen to react with samples and the final volume was 100 μl. The plate was incubated at 37 °C for 10 min and then removed from heat and sat at room temperature for 2.5 h. Then, 200 μl of 0.2 M carbonate stop buffer was added to each well. Fluorescence was determined with emission and excitation filters set at 465 nm and 360 nm, respectively. The values for each time interval were averaged after subtracting the blank.

**Transcriptome analysis**

Xcc cells from overnight culture in NYG medium were collected, washed twice with MMX medium and then transferred to 10 ml fresh MMX medium to a final optical density of 0.3 (600 nm) and incubated till the concentration up to OD600 = 0.6. The total RNA was extracted from the cultures with SV Total RNA Isolation System (Promega). RNA samples were quantified and qualified by Agilent Bioanalyzer (Agilent Technologies). The RNA integrity number (RIN) of total RNA should be greater than 8.0 and the rRNA ratio (23S/16S) should be greater than 1.2. The total RNA samples were digested by RQ DNase I (Promega) with a concentration of 1 U/μg of RNA samples. The RNA samples for transcriptome analysis were prepared according to the manufacturer's manuals (Illumina). Briefly, rRNA was cleaned by Ribo-Zero™ rRNA Removal Kit (Gram-Negative Bacteria) (Epicentre Biotechnologies). After purification, the mRNA was fragmented into small pieces for first strand cDNA synthesis using the fragment agent (divalent cations) under elevated temperature. The synthesized cDNA fragments were added with adapters at their ends by an end repair process. The obtained products were purified and enriched with PCR to create the final cDNA libraries. The quality of these cDNA libraries was assessed using the Agilent Bioanalyzer and ABI Step One Plus Real-Time PCR (Applied Biosystems). The RNAs were sequenced by the Illumina sequencing platform (HiSeq 2000) in Beijing Genomics Institute at Shenzhen (BGI).
Analysis of sequence data

The raw reads generated from the sequencing were cleaned up and mapped to the reference genomic sequence of \textit{Xcc} strain 8004 by SOAP2/SOAP aligner [36]. The expression levels were evaluated by reads per kilobase per million mapped reads (RPKM) [37], which normalizes the reads count to the gene expression level by taking account of the gene length and sequencing depth. The differential expression genes (DEGs) analysis was performed as described by Audic and Clavier [38], in which false discovery rate (FDR) was used to determine the threshold of p-value in multiple tests. In this study FDR < 0.001 was used as the threshold to judge the significance of gene expression difference. RNA sequencing data from four samples (\textit{ΔhrpC}-1, \textit{ΔhrpC}-2, \textit{Xcc} 8004–1 (WT-1), \textit{Xcc} 8004–2 (WT-2)) were grouped into four pairs (\textit{ΔhrpC}-1/WT-1, \textit{ΔhrpC}-1/WT-2, \textit{ΔhrpC}-2/WT-1, and \textit{ΔhrpC}-2/WT-2). The log2 fold change of RPKM of mutant vs. wild type was counted. The average of the log2 fold values of the four pairs was used to assess the differential expression genes with a stringent cutoff value of |log2-fold value| ≥ 1.0 and \( p \) value < 0.01. The RNA sequencing strategy for \textit{ΔrpfG} was the same as \textit{ΔrpfC}.

qRT-PCR analysis

\textit{Xcc} cells from overnight culture in NYG medium were collected, washed twice with MMX medium and transferred to 10 ml fresh MMX medium to a final optical density of 0.3 (600 nm) and incubated till the concentration up to \( \text{OD}_{600} = 0.6 \). The total RNA was extracted from the cultures with SV Total RNA Isolation System (Promega). The PrimeScriptTM RT reagent Kit with gDNA Eraser (Perfect Real Time) (TakaRa) was employed to fulfill the digestion of genomic DNA and the synthesis of cDNA. The obtained cDNA template was diluted to a final concentration of 5 ng/μl and 2 μl aliquot was used for qRT-PCR analysis. 16S rDNA gene was used for normalization in the qRT-PCR analysis. The primer sets for randomly selected ORFs, \textit{hrp} genes, and type III effector genes were listed in Table 2.

Results

Identification of positive regulator candidates affecting \textit{hrpX} expression by \textit{sacB} strategy

The \textit{sacB} gene that encodes a levansucrase in \textit{Bacillus subtilis} has been used as a tool for positive selection [23, 39–41]. The enzyme levansucrase catalyzes transfructorylation
from sucrose to various acceptors, resulting in sucrose hydrolysis and the synthesis of levan, which is toxic to cells. It has been reported that expression of sacB gene in the presence of 5% sucrose in agar medium is lethal to a variety of bacteria including E. coli, Agrobacterium tumefaciens, and Rhizobium meliloti [23]. In this study, we found that similar to these bacteria, Xcc strain 8004 expressing sacB gene could not survive at the same sucrose concentration. Therefore, we used the sacB gene to screen candidates which positively regulate the expression of hrpX. In brief, firstly we constructed a recombinant plasmid pL6hrpXsacB (Table 1) by cloning a sacB gene into the broad host range plasmid pLAFR6 (Table 1), in which the sacB gene was driven by the promoter of hrpX. Then, the plasmid pL6hrpXsacB was transferred from E. coli into Xcc wild type strain 8004 by triparental conjugation. The obtained transconjugant strain 8004/pL6hrpXsacB (Table 1) was mutated by the EZ-Tn5 transposon, followed by selecting mutant colonies on the plates of MMX minimal medium containing 5% sucrose. The principle in this strategy is that strain 8004/pL6hrpXsacB cannot grow on the minimal medium MMX containing 5% sucrose (Fig. 1b), because the expression of the hrpX-promoter-driven sacB gene is lethal to the cells under these conditions. However, the strains with a mutation (i.e., deletion mutant of hrpG, ΔhrpG) impeding the expression of hrpX (i.e. strain ΔhrpG/pL6hrpXsacB) (Fig. 1c) or disrupting the sacB gene and the wild-type strain 8004 as well as the deletion mutant strain ΔhrpG can grow (Fig. 1a and d).

Six mutants (named XB001 to XB006) (Table 1) were obtained in this work. The transposon insertion sites in these mutants were further mapped (see Methods for details), revealing that the mutations lie in the ORFs XC_4007 (XB001), XC_2333 (XB003), XC_1192 (XB004), XC_3951 (XB005) and XC_0124 (XB006), and the intergenic region between the ORFs XC_1510 and XC_1511 (XB002), respectively. Interestingly, the ORF XC_2333 is the rpfC gene. The others were annotated to encode hypothetical proteins (XC_4007 and XC_1511), anti-freeze glycopeptide AFGP related protein (XC_1192), glucosyltransferase (XC_3951), TonB-dependent receptor (XC_0124), and TldD protein (XC_1510), respectively.

RpfC positively regulates the expression of hrpX

As described above, RpfC is a key sensor kinase in rpf/T3SS system. The above result suggests that RpfC may also play a role in the regulation of hrp/T3SS system. To further validate this result, we constructed a deletion mutant of rpfC (named ΔrpfC) and promoter-gusA transcriptional fusion reporter plasmids of Xcc hrpG and hrpX (named pGUSHrpG and pGUSHrpX) (see the Methods for details). The reporter plasmids were then transferred into the rpfC deletion mutant ΔrpfC and the wild-type strain 8004 by triparental conjugation, yielding reporter strains ΔrpfC/pGUSHrpG, ΔrpfC/pGUSHrpX, 8004/pGUSHrpG, and 8004/pGUSHrpX, respectively (Table 1). Subsequently, GUS activities of these strains grown in hrp-inducing minimal medium XCM1 were assayed. The results showed that the GUS activities of the strain ΔrpfC/pGUSHrpX was significantly lower than that of the strain 8004/pGUSHrpX (p = 0.005 by t test) (Fig. 2). Although the GUS activity of strain ΔrpfC/pGUSHrpG was lower than that of strain 8004/pGUSHrpG, their difference was not significant (p = 0.3344 by t test) (Fig. 2). These data suggest that RpfC is involved in positive regulation of the expression of hrpX and the regulation is probably independent of HrpG in the minimal medium XCM1.

To investigate whether RpfC regulates the expression of hrpG and hrpX in plants, the above reporter strains...
were inoculated into the host plant Chinese radish and the GUS activity in the inoculated levels were measured. As shown in Fig. 3, the strain ΔrpfC/pGUShrpX produced significantly lower GUS activity compared to the strain 8004/pGUShrpX, suggesting that RpfC positively regulates the expression of hrpX in planta. Interestingly, the strain ΔrpfC/pGUShrpG also produced significantly lower GUS activity compared to the strain 8004/pGUShrpG (Fig. 3). This indicates that RpfC regulates the expression of hrpG in planta. Taken together, these results imply that RpfC regulates the expression of hrpX in the minimal medium XCM1 as well as in the host plant Chinese radish and influences significantly the expression of hrpG in the host plant tissues but not in XCM1 medium.

**Mutation of rpfC results in a delayed and weakened HR induction**

The above results showed clearly that rpfC positively regulates the expression of the key regulator hrpX of the hrp/T3SS system. To verify whether mutation of rpfC affects the pathogen to induce HR on plants, the mutant strain ΔrpfC and the complemented strain CΔrpfC (Table 1) were tested on Xcc nonhost pepper cultivar ECW-10R (*Capsicum annuum* cv. ECW-10R), which carries the resistance gene Bs1 and has been typically used to test the HR of *Xcc* [33]. The experiment was carried out by infiltrating bacterial suspensions with a cell concentration of OD600 = 0.01 into the plant leaves. Strain ΔavrBs1, an avrBs1-deletion mutant of Xcc, which cannot elicit any HR symptoms on the pepper cultivar [42], was included as a negative control. Eight hours after inoculation, no significant HR phenotype was observed for the mutant strain ΔrpfC, while typical HR symptoms induced by the wild type strain 8004 and the complemented strain CΔrpfC were observed (Fig. 4a). However, the mutant strain ΔrpfC produced visible HR symptoms 16 h after inoculation (Fig. 4a). These results were further substantiated using an electrolyte leakage assay. Both mutants (ΔrpfC and ΔavrBs1) showed significantly decreased electrolyte leakages at 8, 16, and 24 h after inoculation compared to the wild-type strain, although ΔrpfC showed stronger electrolyte leakage than ΔavrBs1 (Fig. 4b). Consistent with the HR symptoms observed, the complemented strain and the wild type induced similar electrolyte leakages 16 h after inoculation (Fig. 4b). Taken together, these results reveal that RpfC is important for Xcc to stimulate a full HR on the nonhost plant pepper cultivar ECW-10R.
RpfC and RpfG regulate the expression of a large set of genes in Xcc 8004

To verify whether mutation of rpfC affects the expression of hrp genes via rpfG in minimal medium, the transcriptome of the mutant strains ΔrpfC and ΔrpfG were determined by RNA deep-sequencing. The mutant strains and the wild type strain 8004 were cultivated in the minimal medium MMX to a cell concentration of OD600 = 0.6–0.8. Total RNA was extracted from the cultures with SV Total RNA Isolation System (Promega). The RNA sequencing was carried out according to the manufacturer’s standard procedure (BGI). Through data analysis (Additional file 1: Table S1), a total of 528 RpfC-regulated genes were identified, among them 328 and 200 were down- and up-regulated, respectively; while 626 RpfG-regulated genes were identified, of which 283 and 343 were down- and up-regulated, respectively. Based on the published gene list of Xcc strain 8004 [4], the products of the RpfC- and RpfG-regulated genes could be grouped into the following 20 functional categories: (I) Nucleotide metabolism, (II) Carbohydrate metabolism, (III) Amino acid and protein metabolism, (IV) Chaperon and peptidases, (V) Fatty acid metabolism, (VI) Extracellular enzymes, (VII) Sugar kinase/transaminase, (VIII) Multidrug resistance and detoxification, (IX) Oxidative stress resistance, (X) Flagellum synthesis and motility, (XI) Hypersensitive reaction and pathogenicity, (XII) Iron uptake, (XIII) Ribosomal

Fig. 4 RpfC is involved in hypersensitive response. a, Hypersensitive response symptoms induced in pepper leaves (Capsicum annuum cv. ECW-10R) by the Xcc strains. Approximately 5 µl bacterial culture (1 × 10^7 CFU/ml) suspended in 10 mM sodium phosphate buffer were infiltrated into the leaf mesophyll tissue with a blunt-end plastic syringe. Pictures of the pepper leaf were taken at 8, 16, and 24 h after infiltration. Three replications were done in each experiment, and each experiment was repeated three times. Results presented are from a representative experiment, and similar results were obtained in all other independent experiments. b, Electrolyte leakage from pepper leaves inoculated with Xcc strains. Results presented are from a representative experiment, and similar results were obtained in other independent experiments.
proteins, (XIV) Transcription regulators, (XV) Dehydrogenase, (XVI) Aerobic and anaerobic respiration, (XVII) Membrane components and transporters, (XVIII) Hypothetical proteins, (XIX) Environmental information processing, (XX) Others (Fig. 5, Additional file 2: Table S2 and Additional file 3: Table S3). To validate the transcriptome data, qRT-PCR was carried out. The result showed that the transcriptional expression of the 24 randomly selected genes, 2 hrp genes [hrpB1 (XC_3011) and hrpF (XC_3025)], and 2 type III effector genes (XC_0241 and XC_4273) was highly consistent with the transcriptome result (Fig. 6). A comparison of the genes regulated by RpfC and RpfG revealed that only 279 of them were regulated by both RpfC and RpfG (Fig. 5). This indicates that the regulons of RpfC and RpfG are not all the same.

**RpfC positively regulates 25 hrp genes, 9 reported T3S effector genes**

The transcriptome result displayed that the expression of all the genes in the hrp cluster (XC_3001-XC_3025) and the regulator hrpX in ΔrpfC mutant cells was significantly ($p \leq 0.01$ by t-test) lower than that in the wild type strain (Table 3). Furthermore, in ΔrpfC mutant cells the expression of the 9 reported T3S effector genes (XC_0241, XC_1553, XC_2004, XC_2081, XC_2602, XC_2995, XC_3160, XC_3177, and XC_4273) was also significantly ($P \leq 0.01$ by t-test) lower than that in the wild type [3, 31, 42–44] (Table 3). However, the expression of hrpG and the global regulator clp in rpf/DSF system was not affected by the mutation of rpfC in the tested conditions (Table 3).

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**Fig. 5** Comparison of RpfC and RpfG regulons. Venn diagrams showing the overlap of genes (a. Total regulated genes. b. hrp genes. c. Type III effector genes) whose expression is upregulated or downregulated in rpfC or rpfG deletion mutant backgrounds.
Notably, the transcriptome analysis revealed that mutation of \textit{rpfG} did not affect the expression of \textit{hrpG}, \textit{hrpX} and \textit{clp} (Table 4), but significantly (\(P \leq 0.01\) by \(t\) test) influence the expression of some \textit{hrp} genes (\textit{XC\_3009} to \textit{XC\_3015}, \textit{XC\_3019}, \textit{XC\_3021}, and \textit{XC\_3025}) and most of the reported T3S effector genes (\textit{XC\_0241}, \textit{XC\_2004}, \textit{XC\_2081}, \textit{XC\_2602}, \textit{XC\_2995}, \textit{XC\_3160}, \textit{XC\_3177}, and \textit{XC\_4273}) (Table 4). Given that RpfC and RpfG compose a two-component regulatory system, it is worthy to further study how they regulate the \textit{hrp} and T3S effector genes. Nevertheless, these results reveal that RpfC positively regulates the expression of \textit{hrp} and T3S effector genes as well as \textit{hrpX} but not \textit{hrpG} and \textit{clp} in the minimal medium MMX.

**Discussion**

The above results demonstrate that the sensor RpfC of the \textit{rpf}/DSF cell-cell signaling system positively regulates the expression of the key regulator \textit{hrpX} of the \textit{hrp}/T3SS system in \textit{Xcc}. Disruption of the \textit{rpfC} gene in \textit{Xcc} strain 8004 caused a significant decrease in the transcription of the \textit{hrp} genes in minimal medium and host plant (Fig. 2, Fig. 3, Table 3, Table 4), resulting in a delayed and weakened HR (Fig. 4). The cell-cell signaling system is generally considered to facilitate gene expression when the bacterial population has reached a sufficient cell density [45]. Almost all of the previous studies on the \textit{rpf}/DSF system of \textit{Xcc} and its regulation in the synthesis of the virulence factors such as extracellular enzymes and EPS were carried out by growing bacterial cells in nutrient rich conditions to allow the bacterium to reach a high cell density. On the contrary, as the expression of \textit{hrp} genes is repressed in nutrient rich media and induced in certain minimal media and plants, almost all of the studies on the \textit{hrp}/T3SS system were carried out in minimal media or plants. The connection between these two systems has been neglected. We were lucky that \textit{rpfC} gene was identified in the mutagenesis screen for \textit{hrpX}-upstream regulatory genes.

Recent evidence suggests that perception of the DSF signal by RpfC leads to activation of RpfG as a phosphodiesterase that degrades cyclic di-GMP. Cyclic di-GMP is a second messenger which can bind to Clp to prevent binding of Clp to the promoters of target genes. The Clp regulator contains an N-terminal cNMP binding domain and a C-terminal DNA-binding domain. The decrease in cyclic di-GMP level by the phosphodiesterase activity releases this inhibition, thus allowing Clp to bind to target promoter DNA sequences and activate target gene expression [13, 14, 46–48]. In a previous transcriptome profiling analysis in \textit{Xcc} strain XC1 cultivated in a nutrient rich medium, it was found that mutation of \textit{clp} affects the transcription of 299 genes. Within these Clp-regulated genes, 260 were up-regulated and 39 down-regulated. The latter genes include 9 \textit{hrp} genes (\textit{hrpB5}, \textit{hrpD5}, \textit{hrcR}, \textit{hrpW}, \textit{hpap}, \textit{hrpB2}, \textit{hrpB7}, \textit{hrpB4}, and \textit{hpal}) but neither \textit{hrpG} nor \textit{hrpX} [15]. These implied that RpfC regulates the expression of the \textit{hrp} genes might via RpfG and the global transcriptional regulator Clp in \textit{Xcc}. However, An and associates found that mutation of \textit{rpfC} or \textit{rpfG} in \textit{Xcc}
Table 3  RpfC positively regulates the expression of hrpX, 25 hrp genes, and 9 T3S effectors

| ID   | Gene name   | Predicted product | Fold change | p value     |
|------|-------------|-------------------|-------------|-------------|
| XC3001 | hpa2        | Hpa2 protein      | -1.967      | 0.006410439 |
| XC3002 | hpa1        | Hpa1 protein      | -3.429      | 5.28933E-05 |
| XC3003 | hrcC        | HrcC protein      | -2.440      | 6.3252E-05  |
| XC3004 | hrcT        | HrpB8 protein     | -2.112      | 0.001062566 |
| XC3005 | hrpB7       | HrpB7 protein     | -2.429      | 3.27619E-06 |
| XC3006 | hrcN        | HrpB6 protein     | -2.184      | 0.000117024 |
| XC3007 | hrpB5       | HrpB5 protein     | -3.356      | 1.38714E-05 |
| XC3008 | hrpB4       | HrpB4 protein     | -2.781      | 0.000112512 |
| XC3009 | hrcJ        | HrcJ protein      | -3.227      | 5.31033E-05 |
| XC3010 | hrpB2       | HrpB2 protein     | -3.152      | 5.78013E-05 |
| XC3011 | hrpB1       | HrpB1 protein     | -3.334      | 3.3299E-06  |
| XC3012 | hrcU        | HrcU protein      | -2.873      | 3.59286E-05 |
| XC3013 | hrcV        | HrcV protein      | -2.871      | 7.99441E-05 |
| XC3014 | hpaP        | HpaP protein      | -2.730      | 0.000117653 |
| XC3015 | hrcQ        | HrcQ protein      | -2.963      | 0.000143701 |
| XC3016 | hrcR        | HrcR protein      | -2.208      | 8.2237E-05  |
| XC3017 | hrcS        | HrcS protein      | -2.664      | 0.000432191 |
| XC3018 | hpaA        | HpaA protein      | -2.373      | 1.30182E-05 |
| XC3019 | hrpD5       | HrpD5 protein     | -2.843      | 2.26091E-05 |
| XC3020 | hrpD6       | HrpD6 protein     | -2.933      | 2.18335E-06 |
| XC3021 | hrpE        | HrpE protein      | -2.076      | 4.12178E-05 |
| XC3022 | hpaB        | HpaB protein      | -2.121      | 1.32695E-08 |
| XC3023 | hrpW        | HrpW protein      | -1.342      | 3.28466E-06 |
| XC3024 | conserved hypothetical protein | -1.376 | 2.43557E-06 |
| XC3025 | hrpF        | HrpF protein      | -2.472      | 3.91605E-06 |
| XC3026 | hrpX        | HrpX protein      | -1.331      | 1.1147E-06  |
| XC3027 | hrpG        | HrpG protein      | -0.564      | 2.03168E-05 |
| XC0052 | avrBs2      | avirulence protein| -0.556      | 0.000371266 |
| XC0241 | xopXccN     | conserved hypothetical protein | -1.713 | 2.02564E-05 |
| XC1553 | avrACxcc8004 | leucin rich protein | -1.796 | 6.64485E-05 |
| XC2004 | avrXccC     | avirulence protein| -1.424      | 0.000257062 |
| XC2081 | avrBs1      | avirulence protein| -1.357      | 0.00061082  |
| XC2602 | avrXccE1    | avirulence protein| -1.458      | 1.49178E-06 |
| XC2994 | xopXccP     | Type III effector protein | -0.626 | 0.000168654 |
| XC2995 | xopXccE1    | Type III effector protein | -1.932 | 2.51053E-06 |
| XC3160 | xopXccR1    | Type III effector protein | -2.954 | 1.98578E-05 |
| XC3177 | xopXccQ     | Type III effector protein | -2.266 | 3.59482E-05 |
| XC3802 | avrXccB     | avirulence protein | -0.449 | 0.000671213 |
| XC4273 | xopXccLR    | leucin rich protein | -1.842 | 3.38357E-07 |
| XC0486 | clp         | CAP-like protein  | 0.091       | 0.000280809 |

Fold change means the value of log2 ratio of RPKM (ΔRpfC/wild type). The differential expression genes were defined with a stringent cutoff value of \(|\text{log}_2\text{fold change}| \geq 1.0\) and \(p\text{ value} < 0.01\)
Table 4 RpfG positively regulates the expression of 10 *hrp* genes, 8 T3S effectors

| ID     | Gene name | Predicted product       | Fold change | p value  |
|--------|-----------|-------------------------|-------------|----------|
| XC3001 | hpa2      | Hpa2 protein            | −0.460      | 0.014094188 |
| XC3002 | hpa1      | Hpa1 protein            | −0.794      | 1.9328E-05 |
| XC3003 | hrcC      | HrcC protein            | −0.748      | 0.000323325 |
| XC3004 | hrcT      | HrpB8 protein           | −0.819      | 0.007692677 |
| XC3005 | hrpB7     | HrpB7 protein           | −0.898      | 0.000925861 |
| XC3006 | hrcN      | HrpB6 protein           | −0.866      | 0.001395029 |
| XC3007 | hrpB5     | HrpB5 protein           | −0.422      | 0.002457912 |
| XC3008 | hrpB4     | HrpB4 protein           | −0.604      | 0.000177562 |
| XC3009 | hrcJ      | HrcJ protein            | −1.370      | 0.00105572 |
| XC3010 | hrpB2     | HrpB2 protein           | −1.189      | 0.000499769 |
| XC3011 | hrpB1     | HrpB1 protein           | −2.031      | 0.000552365 |
| XC3012 | hrcU      | HrcU protein            | −1.364      | 1.2705E-05 |
| XC3013 | hrcV      | HrcV protein            | −1.251      | 0.000455787 |
| XC3014 | hpaP      | HpaP protein            | −1.270      | 0.000271481 |
| XC3015 | hrcQ      | HrcQ protein            | −1.055      | 0.000255553 |
| XC3016 | hrcR      | HrcR protein            | −0.969      | 0.003879682 |
| XC3017 | hrcS      | HrcS protein            | −0.999      | 0.032254632 |
| XC3018 | hpaA      | HpaA protein            | −0.511      | 0.000910631 |
| XC3019 | hrpD5     | HrpD5 protein           | −1.198      | 0.000505121 |
| XC3020 | hrpD6     | HrpD6 protein           | −1.141      | 0.000534484 |
| XC3021 | hrpE      | HrpE protein            | −1.138      | 0.000719991 |
| XC3022 | hpaB      | HpaB protein            | −0.589      | 0.000803494 |
| XC3023 | hrpW      | HrpW protein            | −0.214      | 9.24647E-05 |
| XC3024 | conserved hypothetical protein | conserved hypothetical protein | −0.621 | 0.000308403 |
| XC3025 | hrpF      | HrpF protein            | −2.360      | 0.000402749 |
| XC3026 | hrpX      | HrpX protein            | 0.034       | 4.24498E-05 |
| XC3077 | hrpG      | HrpG protein            | −0.105      | 0.000180844 |
| XC0052 | avrBs2    | avirulence protein      | 0.037       | 0.002116633 |
| XC0241 | xopXccN   | conserved hypothetical protein | −1.272 | 0.000227566 |
| XC1553 | avrACc8004 | leucin rich protein     | −0.942      | 0.000122936 |
| XC2004 | avrXccC   | avirulence protein      | −1.135      | 0.00359996 |
| XC2081 | avrBs1    | avirulence protein      | −1.786      | 0.002769123 |
| XC2602 | avrXccE1  | avirulence protein      | −1.512      | 0.000120947 |
| XC2994 | xopXccP   | Type III effector protein | −0.970  | 0.001806466 |
| XC2995 | xopXccE1  | Type III effector protein | −1.246  | 0.000429812 |
| XC3160 | xopXccR1  | Type III effector protein | −2.452  | 0.000264107 |
| XC3177 | xopXccQ   | Type III effector protein | −2.164  | 0.001441317 |
| XC3802 | avrXccB   | avirulence protein      | −0.562      | 0.002544406 |
| XC4273 | xopXccLR  | leucin rich protein     | −1.251      | 0.00044297 |
| XC0486 | cIP       | CAP-like protein        | 0.199       | 0.000155663 |

Fold change means the value of log2 ratio of RPKM (ΔΔrfpG/wild type). The differential expression genes were defined with a stringent cutoff value of |log2-fold change| ≥ 1.0 and p value < 0.01.
strain 8004 grown in the nutrient rich medium NYG did not affect the expression of hrp genes [49]. Our RNA sequencing data demonstrated that in minimal medium, RpfC positively regulates the expression of nearly all the hrp genes (Table 3) and RpfG controls some of the hrp genes (Table 4). These results indicate that RpfC and RpfG have different effects on the expression of the hrp genes in Xcc strain 8004 when grown in nutrient-rich and nutrient-deficient conditions. Our data also displayed that in minimal medium RpfC regulates the expression of hrfX but not hrfG and RpfG does not regulate the expression of both hrfG and hrfX (Table 3, Table 4). These results suggest that RpfC activate the expression of hrfX in minimal medium via neither RpfG nor HrpG. However, mutation of rpfC significantly reduced the expression of not only hrfX but also hrfG in planta (Fig. 3). This implies that RpfC regulates the hrf genes through different manners in minimal medium and host plants.

As mentioned above, it is known that the core regulatory mechanism in Xcc rpf/DSF quorum sensing system is RpfC-RpfG-c-di-GMP-Clp cascade. However, our transcriptome result showed that the regulons of RpfC and RpfG in the minimal medium MMX are not all the same. Similarly, the regulons of RpfC and RpfG of Xanthomonas citri subsp. citri in nutrient rich medium are also different [50]. These findings suggest that RpfC may regulate a number of genes independent of RpfG. Our data presented in this work show that RpfC may employ an undefined pathway other than the RpfC-RpfG-c-di-GMP-Clp cascade to regulate the expression of the hrf key regulator HprX in the minimal medium MMX. To further dissect how RpfC affects the expression of hrfX will be commendable. Interestingly, RpfC controls the expression of hrfG in host plants (Fig. 3). This suggests that the regulation net between the rpf/DSF and hrf/T3SS systems are rather complex. To further uncover this issue will be valuable.

Conclusions
In this work, we found that mutation of the gene encoding the sensor RpfC of the rpf/DSF system significantly reduced the expression of hrfX, the key regulator of the hrf/T3SS system. Here, we provide evidences to demonstrate that RpfC positively regulates the expression of hrfX independent of RpfG, the cognate response regulator of RpfC, showing a complex regulatory network linking the rpf/DSF and hrf/T3SS systems.

Additional files

Additional file 1: Table S1. RNA sequencing detail raw data. (XLS 8055 kb)

Additional file 2: Table S2. Functional groups of RpfC-regulated genes. (DOCX 19 kb)

Additional file 3: Table S3. Functional groups of RpfG-regulated genes. (DOCX 20 kb)
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