The Post-transcriptional Regulator \textit{rsmA/csrA} Activates T3SS by Stabilizing the 5' UTR of \textit{hrpG}, the Master Regulator of \textit{hrp/hrc} Genes, in \textit{Xanthomonas}

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Abstract

The RsmA/CsrA family of the post-transcriptional regulators of bacteria is involved in the regulation of many cellular processes, including pathogenesis. In this study, we demonstrated that RsmA not only is required for the full virulence of the phytopathogenic bacterium \textit{Xanthomonas citri} subsp. \textit{citri} (XCC) but also contributes to triggering the hypersensitive response (HR) in non-host plants. Deletion of \textit{rsmA} resulted in significantly reduced virulence in the host plant sweet orange and a delayed and weakened HR in the non-host plant \textit{Nicotiana benthamiana}. Microarray, quantitative reverse-transcription PCR, western-blotting, and GUS assays indicated that RsmA regulates the expression of the type 3 secretion system (T3SS) at both transcriptional and post-transcriptional levels. The regulation of T3SS by RsmA is a universal phenomenon in T3SS-containing bacteria, but the specific mechanism seems to depend on the interaction between a particular bacterium and its hosts. For Xanthomonads, the mechanism by which RsmA activates T3SS remains unknown. Here, we show that RsmA activates the expression of T3SS-encoding \textit{hrp/hrc} genes by directly binding to the 5' untranslated region (UTR) of \textit{hrpG}, the master regulator of the \textit{hrp/hrc} genes in XCC. RsmA stabilizes \textit{hrpG} mRNA, leading to increased accumulation of HrpG proteins and subsequently, the activation of \textit{hrp/hrc} genes. The activation of the \textit{hrp/hrc} genes by RsmA via HrpG was further supported by the observation that ectopic overexpression of \textit{hrpG} in an \textit{rsmA} mutant restored its ability to cause disease in host plants and trigger HR in non-host plants. RsmA also stabilizes the transcripts of another T3SS-associated \textit{hrpD} operon by directly binding to the 5' UTR region. Taken together, these data revealed that RsmA primarily activates T3SS by acting as a positive regulator of \textit{hrpG} and that this regulation is critical to the pathogenicity of XCC.

Introduction

Pathogenic bacteria belonging to the genus \textit{Xanthomonas} cause diseases in many economically important plants throughout the world. The virulence of these bacteria depends on a type 3 protein secretion system (T3SS) [1,2,3]. T3SS mediates the translocation of bacterial effector proteins into the host cell, where they may interfere with host metabolic pathways and/or suppress plant defense reactions [4,5,6,7]. Genes encoding the T3SS are referred to as Hypersensitive Response and Pathogenicity (\textit{hrp}) genes because \textit{hrp} mutants lost the abilities to trigger the HR in non-host plants and pathogenesis in host plants [8]. In Xanthomonads, the \textit{hrp} gene cluster contains at least 22 genes, nine of which are highly conserved and therefore have been termed \textit{hrc} (\textit{hrp} conserved) genes [4]. The expression of the \textit{hrc} cluster of genes in \textit{Xanthomonas} has been shown to be activated in planta and in the minimal medium XVM2 by the transcriptional regulators HrpG and HrpX [9,10]. HrpG is a response regulator belonging to the OmpR family of two-component system response regulators that contains an N-terminal response receiver (RR) domain and a C-terminal DNA-binding motif [10,11]. Recently, a putative cognate sensor histidine kinase was reported for HrpG in \textit{Xanthomonas campestris}, but further studies are needed to identify the activation signal and whether homologs of this sensor kinase are functional in other \textit{Xanthomonas} species [1,7,12]. Phosphorylated HrpG is predicted to activate the expression of \textit{hrpX}, which encodes an AraC-type regulator [11]. HrpX binds to a cis-regulatory element, the plant inducible promoter (PIP) conserved motif (TTCGC-N15-TTCGC) or a less-conserved PIP-like motif (TTCGC-N7-TTCGT), which is present in the promoter regions of four operons (\textit{hrpB}, \textit{hrpC}, \textit{hrpD} and \textit{hrpE}) within the \textit{hrc} cluster (Fig. S1), and a set of genes involved in the virulence of \textit{Xanthomonas} [9,13,14]. Likewise, HrpG and HrpX homologs have been reported to mediate the expression of genes in the \textit{hrc} cluster of the plant pathogenic bacteria \textit{Ralstonia solanacearum} and the animal pathogen \textit{Burkholderia pseudomallei} [15,16]. The trigger signal and post-transcriptional regulation of HrpG/HrpX-dependent signaling pathway, which is different from that in \textit{Erwinia carotovora} and \textit{Pseudomonas} spp., denominated type I regulatory system, are poorly understood [7,17,18].

Further studies in \textit{Xanthomonads} indicate that the post-transcriptional regulator \textit{rsmA} (repressor of secondary metabolism) positively regulates pathogenicity [19,20]. RsmA belongs to a conserved family of RNA-binding proteins that were initially identified as repressors of carbon metabolism [carbon storage regulator (CsrA)] [21,22]. Members of the RsmA/CsrA family are...
Author Summary

Pathogenic bacteria demonstrate sophisticated capacity to regulate gene expression to meet requirements of living in different environmental niches, including in the hosts. The activation of the Type 3 secretion system (T3SS) genes in response to the host environment is under the control of several factors, such as the post-transcriptional regulator RsmA/CsrA. Here, we show that RsmA contributes to the pathogenicity of Xanthomonas citri in host plants and the HR-triggering activity in non-host plants by regulating the expression of T3SS-encoding hrp/hrc genes. RsmA directly interacts with the 5’ UTRs of hrpG and hrpD mRNAs, which leads to increased HrpG protein levels by stabilizing the hrpG transcript. Further, overexpression of hrpG in an rsmA mutant restored its pathogenicity and ability to cause HR. The deletion of rsmA did not affect the phosphorylation of HrpG, which is also required for T3SS activation. This work provides mechanistic insights for the first time into RsmA-mediated regulation of T3SS gene expression by acting as a positive regulator of hrpG at the post-transcriptional level.

widely distributed among eubacteria and control various traits including biofilm formation, motility, carbon flux, secondary metabolism, quorum-sensing, and virulence to animal and plant hosts [22,23,24,25,26,27,28]. RsmA/CsrA has been shown to regulate the T3SS genes of many animal and plant pathogenic bacteria including Pseudomonas aeruginosa [29], Salmonella enterica Serovar Typhimurium [28], Erwinia carotovora [30,31], Dickeya dadantii [32], and Xanthomonas spp. [19,20]. In X. campestris pv. campestris and X. oryzae pv. oryzae, deletion of an rsmA homolog resulted in a complete loss of virulence in host and a loss of HR induction in non-host plants, impairment of endoglucanase production, and enhanced biofilm formation [19,20]. While T3SS genes have been shown to be regulated by RsmA homologs in many animal and plant pathogenic bacteria, the mechanistic understanding of RsmA-mediated regulation of T3SS genes in Xanthomonads remained unknown.

RsmA can bind to the 5’ untranslated regions (UTRs) of specific mRNAs by recognizing a wide variety of sequences containing the conserved trimucleotide motif GGA in the loop structures [22,33,34,35]. By directly binding to the target mRNAs, RsmA can either down- or up-regulate the expression of target genes or inhibit translation by blocking the access of the ribosome to the mRNAs by recognizing a wide variety of sequences containing the GGA motif. Inhibiting translation by blocking the access of the ribosome to the mRNAs can either down- or up-regulate the expression of target genes or proteins [22,33,34,35]. By directly binding to the target mRNAs, RsmA/CsrA has been shown to inhibit translation by blocking the access of the ribosome to the mRNAs by recognizing a wide variety of sequences containing the GGA motif. Inhibiting translation by blocking the access of the ribosome to the mRNAs can either down- or up-regulate the expression of target genes or proteins.

how RsmA activates the hrp/hrc genes of Xanthomonas citri subsp. citri (XCC), which causes the important citrus canker disease throughout the world [45]. In this paper, we present a model in which RsmA positively regulates the hrp/hrc genes in Xanthomonas, and discuss the implications of these findings in plant-host-pathogen interactions.

Results

RsmA plays a central role in the activation of the pathogenicity and HR of XCC

The rsmA gene of XCC (XAC1743, GenBank accession number NP_642074) consists of 213 bp nucleotides and encodes a protein comprising 70 amino acids [46]. RsmA of XCC displays 74% identity with E. coli K12 CsrA (NP_417176.1), 93% with X. campestris pv. campestris strain 8004 RsmA (AAV49556), and 100% with X. oryzae pv. oryzae strain 13751 XOO_2760 (NC_007705.1).

The sequence alignment of Xanthomonas RsmA with homologous proteins exhibited many highly conserved residues in its primary structure [19]. One notable exception is the presence of nine additional residues in the carboxy-terminal region of RsmA of Xanthomonas spp. that do not exist in RsmA homologs in Enterobacteria, Pseudomonas spp. and Erwinia spp. (Fig. S2A) [30,47,48]. An unusual C-terminal extension is also present in an RsmA homolog in Sinorhizobium meliloti (RsmAsm) which is 81% identical to XCC RsmA [49]. Deletion of the extended C-terminal domain of RsmAsm affects its cellular concentration, but enhances its relative RNA-binding activity. Interestingly, computational analysis of the predicted secondary structure of XCC RsmA by Phyre [50] revealed that a unique α-helix motif is formed between residues 44–60 (Fig. S2A), in contrast to RsmA homologs from other species with α-helix motifs restricted to residues 44–53 [33,34]. This difference could be caused by the extended C-terminal domain of RsmAsm, suggesting that RsmA regulation involves a novel, as-yet-unidentified mechanism.

To study the contribution of rsmA to the pathogenicity of XCC, we employed an allelic exchange protocol to construct the ΔrsmA mutant of XCC strain 306 containing an in-frame deletion of rsmA codons 5–66 (Table S1). The deletion of rsmA was confirmed by PCR, and the complementation with wild-type rsmA restored the virulence in the host plants (Fig. 1).

While infection of the host plant sweet orange (Citrus sinensis) with wild-type XCC strain resulted in clear canker disease symptoms, the ΔrsmA strain caused less hypertrophy and hyperplasia and failed to produce water-soaking and necrosis symptoms in the susceptible citrus host (Fig. 1A). Consistent with the reduced symptom development caused by the ΔrsmA mutant, our results also indicated that rsmA is essential for the proliferation...
of XCC in host plants (Fig. 1B). The deletion of rsmA impaired the growth of XCC inside the host, whereas the complemented strain multiplied to the same level as wild-type (Fig. 1B). These results are consistent with previous studies showing the requirement of rsmA homologs for the virulence of X. campestris pv. campestris and X. oryzae pv. oryzae in their specific hosts [19, 20]. In addition, it was observed that rsmA contributes to HR induction in the non-host tobacco plant (Nicotiana benthamiana) (Fig. 1C).

To determine whether mutation in rsmA has any effect on the growth of XCC in media, we examined the growth of the rsmA mutant strain (ΔrsmA), the wild-type strain XCC and the complemented strain pUFBrsmA in the minimal medium XVM2 [56, 57]. The results showed that the ΔrsmA and wild-type XCC strains grew to similar levels in the XVM2 medium (Fig. 1D), indicating that rsmA is not required for the growth of XCC in the XVM2 minimal medium. We also observed an increase in RsmA protein levels in the mid-log and late-log growth stages of wild-type XCC carrying the pUFBrsmA-flag construct (Fig. 1D).

RsmA Activates T3SS by Stabilizing 5′ UTR of hrpG

The significant contribution of RsmA to the pathogenicity and HR-triggering activity of XCC prompted us to investigate its regulatory effect on the hrp/hrc genes by confirming whether RsmA controls the expression of T3SS genes as has been observed in other pathogenic bacteria. The expression of hrp/hrc genes in Xanthomonads has been reported to be repressed in nutrient-rich media but strongly induced in planta and in the XVM2 medium [10, 56]. A previous microarray analysis showed that the whole XCC hgp gene cluster, which contains 24 genes (XAC0393 to XAC0417) [Fig. S1], was down-regulated in hgpG and hgpX mutants of XCC grown in XVM2 medium [9].
To test whether RsmA regulates the expression of hrp/hrc genes in XCC, microarray analyses were conducted to compare the gene expression of the ΔrsmA mutant with that of wild-type XCC strain 306 grown in XVM2 medium to OD600 nm = 0.5. In this study, false discovery rate (FDR) = 0.05 and absolute value of log2-fold change = 1 (equivalent to a fold change of 2) were used as the cutoff values. Compared to wild-type XCC strain 306, a total of 82 genes were down-regulated and 88 genes were up-regulated in the ΔrsmA mutant (Table S2). Overall, 23 genes in the hrp cluster and 16 genes encoding putative T3SS effectors were down-regulated in the ΔrsmA mutant (Table S2). The microarray data were validated by quantitative reverse-transcription PCR (qRT-PCR) of 11 hrp genes. Although we observed differences in the amplitude of fold changes determined using the two techniques, the general trends in gene expression were consistent for all 11 genes that were tested (Table 1).

### Table 1. rsmA regulation of the expression of the genes in the hrp cluster of Xanthomonas citri subsp. citri.

| Locus tag | Gene name | Log2 ratio of fold change (ΔrsmA/WT)* | Log2 ratio of fold change (pBRArsmA/pBRA-XCC)§ | qRT-PCR (ΔrsmA/WT)* | Product description |
|-----------|-----------|----------------------------------------|-----------------------------------------------|----------------------|-------------------|
| XAC0394   | hrpF      | −1.23                                  | 2.79                                          | -                    | HrpF              |
| XAC0396   | hpaB      | −1.13                                  | 3.31                                          | -                    | HpaB              |
| XAC0397   | hrpE      | −1.44                                  | 2.65                                          | −3.20                | HrpE              |
| XAC0398   | hpdO6     | −1.30                                  | 2.87                                          | −3.04                | HpdO6             |
| XAC0399   | hpdO5     | −1.56                                  | 3.50                                          | −2.76                | HpdO5             |
| XAC0400   | hpaA      | −1.25                                  | 3.58                                          | -                    | HpaA              |
| XAC0401   | hrcS      | −1.31                                  | 3.62                                          | −2.94                | HrcS              |
| XAC0402   | hrcR      | −1.41                                  | 3.53                                          | -                    | HrcR              |
| XAC0403   | hrcQ      | −1.73                                  | 3.98                                          | −3.32                | HrcQ              |
| XAC0404   | hpaP      | −1.10                                  | 3.37                                          | -                    | HpaP              |
| XAC0405   | hrcV      | −1.26                                  | 3.35                                          | -                    | HrcV              |
| XAC0406   | hrcU      | −1.40                                  | 3.78                                          | −2.82                | HrcU              |
| XAC0407   | hpbB1     | −1.37                                  | 3.48                                          | −3.14                | HpbB1             |
| XAC0408   | hpbB2     | −1.32                                  | 3.92                                          | −2.65                | HpbB2             |
| XAC0409   | hpcJ      | −1.82                                  | 3.91                                          | -                    | HpcJ              |
| XAC0410   | hpbB4     | −1.75                                  | 4.05                                          | -                    | HpbB4             |
| XAC0411   | hpbB5     | −1.46                                  | 4.12                                          | -                    | HpbB5             |
| XAC0412   | hrcN      | −1.23                                  | 3.78                                          | -                    | HrcN              |
| XAC0413   | hpbB7     | −1.17                                  | 4.02                                          | -                    | HpbB7             |
| XAC0414   | hrcT      | −1.00                                  | 3.96                                          | -                    | HrcT              |
| XAC0415   | hrcC      | −1.06                                  | 2.51                                          | −2.84                | HrcC              |
| XAC0416   | hpa1      | −1.72                                  | 3.28                                          | −3.12                | Hpa1              |
| XAC0417   | hpa2      | −1.13                                  | 2.53                                          | −2.48                | Hpa2              |
| XAC1265   | hpg       | −1.42                                  | 2.02                                          | −2.27                | Hpg               |
| XAC1266   | hpx       | −1.38                                  | 2.11                                          | −2.32                | Hpx               |

*The Log2-fold change of each gene was derived by comparing the rsmA mutant versus wild-type (p<0.05; Log2≥1).

The Log2-fold change of each gene was derived by comparing the ΔrsmA mutant strains carrying the construct pBRArsmAxc and the empty plasmid pBRA. Transcriptome analyses were performed with four independent biological samples for each condition.

doi:10.1371/journal.ppat.1003945.t001
virulence, the expression of T3SS genes was significantly activated by the overexpression of RsmA in the Δsmc4 mutant (Table 1). Taken together, these data confirmed that smc4 contributes to virulence by activating the expression of htp/hrc genes.

**Deletion of smc4 reduces protein levels of T3SS components**

To investigate whether the deletion of smc4 also caused a reduction in the protein levels of T3SS, we performed Western-blotting experiments to evaluate the abundances of HrcU, HrpB1, HrpB2 and HrpD6 in total cell extracts of wild-type XCC strain 306, the Δsmc4 mutant, and the complementation strain grown in the XVM2 medium. Based on the immunoblotting results, the relative protein levels in the Δsmc4 mutant was estimated in reference to the wild-type strain and normalized according to two unsppecific protein bands also recognized by the polyclonal antibodies (Figs. 2A, 2B and 2C). The protein levels of HrcU, HrpB1, HrpB2, and HrpD6 were significantly reduced to 14%, 40%, 3% and 10%, respectively, in the Δsmc4 mutant (Figs. 2A, 2B and 2C). Interestingly, the antibodies generated against HrcU recognized two peptides of approximately 28 and 10 KDa, respectively (Fig. 2C). Indeed, HrcU can undergo autocatalytic cleavage between the asparagine and the proline residues of a conserved NPTH motif (amino acids 264–267), resulting in a reorientation of the PTH loop, which is required for the secretion of late T3SS substrates [58,59]. The relative protein levels of HrcU, HrpB1, HrpB2 and HrpD6 were restored by the expression of smc4 in the complemented strain (Figs. 2A, 2B and 2C).

The positive regulation of htp/hrc genes by RsmA in XCC was further investigated using translational fusion constructs with a gusA reporter gene [60,61]. The translational fusion constructs harbor the native promoter, the 5′ leader region and at least the first three codons of the coding DNA sequences of hgpG, hgpX, hgpA genes, as well as hgbP, hgpD and hgpE operons fused in-frame to the ORF (open reading frame) of gusA (Fig. 2D). To differentiate transcriptional and post-transcriptional regulation, we investigated whether replacing the original promoters of the htp/hrc genes by constitutively expressed Plac promoter affects the GUS activities in wide type and the Δsmc4 mutant strains (Fig. 2E). Results of the GUS activities demonstrated that the Δsmc4 mutation significantly affected the expression of all hgp genes from their native promoters, but it specially affected the expression of hgpG and hgpD under the control of Plac (Figs. 2D, 2E). Taken together, these results indicated that RsmA regulates the expression of htp/hrc genes at the transcriptional level, and may also control hgpG and hgpD expression at the post-transcriptional level.

**Mapping of the 5′-UTR sequences of htp/hrc transcripts**

Previous studies have suggested that RsmA binds to the consensus sequence, ACARGGAUG, with the GGA motif representing the most conserved nucleotides [35,62]. RsmA can bind to the 5′-UTR of a specific mRNA and negatively or positively regulate the transcript stability and thus the translation [22]. Because RsmA activates all htp/hrc genes including hgpG and hgpX (Table 1), the two master regulators of htp/hrc genes, we hypothesized that RsmA exerts its regulation of the htp/hrc genes via hgpG, the upmost in the regulatory hierarchy. Alternatively, RsmA could bind to the 5′ UTR of each individual transcription unit of the htp cluster.

The transcriptional initiation sites of the genes hgpG, hgpX, hgpF, and also of the operons hgpB, hgpD, hgpE and hgpF in XCC were determined by rapid amplification of 5′-xDNA Ends (5′ RACE) (Fig. 3A). The RACE PCR products were then sequenced to identify specific nucleotides, indicated by arrows in the Figure 3B, as the transcription start sites of individual transcription units. The analysis of the 5′ RACE results allowed us to determine the PIP-box promoters, -35 and -10 regions, leader sequences and the first codon for each htp/hrc transcript in XCC (Fig. 3B).

The PIP-box promoters and leader sequences for the htp/hrc transcripts were previously experimentally identified in X. campesiris pv. vesicatoria and X. oryzae pv. oryzae [4,13,46,63,64]. Our results confirmed that the htp/hrc transcriptional start sites identified in XCC are similar to those reported in X. campesiris pv. vesicatoria [4,57,63,65]. To identify the putative RsmA binding sites in the htp/hrc transcripts, we analyzed the presence of the GGA motifs in the 5′ leader sequences of the hgpB, hgpC, hgpD, hgpE, hgpF, hgpG, and hgpX transcripts of XCC (Fig. 3B). We were able to find putative RsmA binding sites in the 5′ leader sequences of the hgpC, hgpD, hgpE and hgpG transcripts. No GGA motifs were found in the 5′ UTRs of hgpB, hgpF or hgpX (Fig. 3B).

**RsmA directly interacts with the 5′ UTRs of hgpG and hgpD**

To experimentally determine whether RsmA of XCC directly binds to the GGA motifs identified in the leader sequences of hgpC, hgpD, hgpE and hgpG transcripts, RNA gel mobility shift assays were performed. RNA gel mobility shift assays were also conducted to test whether RsmA binds to the leader sequences of hgpB, hgpF and hgpX, which do not contain putative RsmA binding sites using RNA oligonucleotides designed based on the predicted secondary structures of the hgpB, hgpF and hgpX 5′ leader sequences by Mfold analysis (Table S4) [66].

The recombinant protein 6HisRsmA was purified by nickel affinity chromatography and tested for binding to a 3′-end-biotin-labeled R9-43 probe, which was previously identified as an E. coli RsmA high-affinity ligand (Table S4) [35]. Our results demonstrated that the XCC 6HisRsmA was able to promote a mobility shift of the 3′-biotin-labeled R9-43 RNA probe (Fig. 4A). We then performed the competition assay by adding unlabeled R9-43 RNA in the reaction, which led to a reduction in the intensity of the shifted band, confirming the specificity of the 6HisRsmA/R9-43 interaction (Fig. 4A).

Next, gel mobility shift analyses of the interactions between 6HisRsmA and biotinylated htp/hrc leader RNA probes were performed at a concentration at least ten-fold lower than the lowest protein concentration used in the binding reactions as in Yakhnin et al. [67]. This enables us to assume that the concentration of free 6HisRsmA remained constant during the binding reaction [34,35]. Interestingly, the recombinant protein 6HisRsmA caused mobility shift in the hgpG and hgpD biotinylated transcripts (Fig. 4B). 6HisRsmA did not interact with the hgpC or hgpF transcripts, which also contain potential GGA motifs in the 5′ untranslated mRNA sequences, or with hgpB, hgpF or hgpX, which do not contain GGA motifs in their 5′ UTRs (Figs. 4B and 4C). The biotinylated RNA probe R9-43 was used as a positive control in these assays (Fig. 4C).

Furthermore, the specificities of RsmA-hgpG and RsmA-hgpD 5′ UTRs interactions were investigated by performing competition experiments using the unlabeled specific competitor R9-43 (Fig. 4B). Also, the RsmA-hgpG 5′ UTR interaction was examined by gel mobility shift using two different biotinylated RNA oligonucleotides hgpG1 and hgpG2 (Table S4), which bear two potential RsmA binding sites found in the hgpG leader sequence. The recombinant protein 6HisRsmA was able to retard the hgpG2 RNA probe (carrying the second GGA motif of the hgpG leader sequence), but not the hgpG1 RNA probe which carries the first GGA motif (Fig. 4C).

The fraction of bound hgpG2 increased when we raised the 6HisRsmA concentration in the binding reactions (Fig. 4D). Using
the biotin-labeled hrpG2 probe, only one complex was observed with 0.065 M of 6HisRsmA, and essentially most of the starting RNA was shifted with 1.2 M of 6HisRsmA. The binding curves, Hill coefficient (n), and apparent equilibrium binding constant (Kd) for the 6HisRsmA-hrpG2 RNA interaction were estimated by a nonlinear least-squares analysis using the average pixel values of the shifted bands determined with the ImageJ software [68,69,70] in three independent experiments, as described previously [70,71]. The apparent Kd value was calculated as 0.18 ± 0.2 M 6HisRsmA (Fig. 4D). This value is around 3.5-fold and 7.5-fold higher than the Kd values of E. coli RsmA/CsrA when interacting with the 5′ leader regions of cstA (Kd ~40 nM) and flhDC or pgaA (Kd ~21 nM), respectively [44,70,72].

The RsmA binding site predicted within hrpG2-AGAUG-GAUUU has an 80% nucleotide identity with the RsmA target site in the high-affinity ligand sequence R9-43-ACARGGAUGU-. As the interaction of RsmA-RNA depends on RNA sequence and secondary structure and because hrpG1 and hrpG2 RNA probes bear only partial sequences of the hrpG leader region, our data could not rule out the possibility that RsmA also can interact with the potential binding site 1 in the hrpG-5′-UTR.

**Figure 2. RsmA regulates protein levels of T3SS in X. citri subsp. citri.** Immunoblotting analyses of the total protein extracts of the wild-type (WT), the rsmA mutant (ΔrsmA) harboring the empty plasmid pUFR047 and the complemented strain (pUFRrsmA) are shown. Bacterial cells were grown in the XVM2 medium and collected at OD600 nm = 0.5. Proteins were separated by SDS-PAGE and transferred to a nitrocellulose membrane. The blots were probed with A) HrpB1, B) HrpD6 or C) HrcU and HrpB2 polyclonal antibodies, respectively. Protein-A conjugated with horseradish peroxidase was used to detect the blots. Beneath the panels are presented the values of the relative levels of detected proteins in rsmA mutant and complemented strains which were estimated according to wild-type results. The estimated values were normalized with the values obtained to unspecific protein bands also recognized by the antibodies. D) and E) GUS assays using translational fusion constructs. The different constructs used in this assay are represented by diagrams bellow of the graphics. D) Wild-type and rsmA mutant cells harboring plasmid-borne promoterless gusA in-frame fused to the native promoters and the first codons of the hrp genes. E) Wild-type and rsmA mutant cells transformed with translational fusions driven by the constitutive Plac promoter. Values presented are means ± standard deviations of three independent experiments. * represents the significant difference between the wild-type and ΔrsmA values by using ANOVA. The GUS assay was repeated twice with similar results.

doi:10.1371/journal.ppat.1003945.g002
experiment with unlabeled in vitro transcribed 5’-UTR of hrpG transcript. The concentrations of 6HisRsmA and the biotinylated hrpG transcript were maintained at 0.5 μM and 6.25 nM, respectively, but the concentrations of unlabeled hrpG transcript were ranged from 0 to 1 μM. The unlabeled in vitro transcribed 5’-UTR of the hrpG transcript was able to compete for 6HisRsmA binding as indicated by the disappearance of the mobility shift at higher concentrations of the competitor (Fig. 5A). Also, the nonspecific yeast tRNA was added to all reactions to verify the specificity of the RsmA-hrpG transcript interaction. These results strengthen our hypothesis that RsmA binds specifically to the leader sequence of hrpG mRNA in XCC.

To determine whether the potential RsmA-binding sites were responsible for the observed mobility shift, we used mutated hrpG transcripts in which the GGA binding sites (BS1 and BS2) were mutagenized to TTA. Interestingly, the hrpG transcript mutated in the potential binding site 1 (BS1) did not prevent the mobility shift by 6HisRsmA (Fig. 5B). However, a significant reduction in the mobility shift was observed when the BS2 of the hrpG transcript was changed to TTA (Fig. 5B). In addition, deletion of a 23 nt fragment containing the GGA motif in the BS2 [ten nt were upstream and the other ten nt were downstream of the GGA motif] led to impairment of the mobility shift of the biotinylated hrpG transcript (Fig. 5B).

Furthermore, different fragments of the hrpG leader sequence were synthesized by in vitro transcription, and after biotinylation they were incubated with 6HisRsmA (0.5 μM) to verify binding activity. We did not observe mobility shift of the hrpG1-81 transcript carrying BS1 of the hrpG. In contrast, we observed a shift for the biotinylated hrpG54-189 transcript, although it is weaker (about 8-fold less) than that of the full length hrpG1-189 transcript (Fig. 5C). Consistently, the addition of the unlabeled hrpG1-189 transcript at high concentrations in the reactions impaired the binding of both hrpG1-189 and hrpG54-189 with 6HisRsmA (Fig. 5C). These results suggest that not only the sequence, but also the secondary structure of the hrpG transcript are critical for its interaction with RsmA.

Effects of RsmA on hrpG and hrpD mRNA stability

As was demonstrated for flhDC in E. coli, the interaction of RsmA with the leader region of hrpG suggests that RsmA may...
stabilize and protect hrpG transcripts against 5’-end-dependent RNase E cleavage in XCC [44,73,74]. Previous studies revealed that differences in the steady-state levels of flhDC and glgC transcripts between E. coli wild-type and rsmA mutant strains are resulted from the effect of RsmA on the rates of mRNA decay [43,44,75]. To determine whether the stability of the hrpG and hpdD transcripts are affected by rsmA in vivo, the abundances of the hrpG and hpdD transcripts and the 16S transcript as a control were analyzed by RT-PCR after the addition of 10 μg/μL ciprofloxacin (Sigma, USA) in XCC cell cultures (Fig. 6A). Ciprofloxacin targets DNA gyrase and topoisomerase IV on DNA, forming ternary complexes that block the movement of replication forks and transcription complexes [76,77]. We used ciprofloxacin in this assay because Xanthomonas spp. exhibit resistance to rifampicin [78,79]. The relative abundance of the hrpG and hpdD transcripts was estimated from the specific PCR products on the agarose gel by calculating the average pixel values of the bands, subtracting the background, and determining the area of the bands by integration in the ImageJ software [68,69,71]. The calculated average pixel values of hrpG and hpdD were normalized based on the pixel value of the corresponding 16S amplified band, the transcript levels of which did not vary in response to the treatment. The hrpG and hpdD transcripts were at least two-fold more stable in wild-type XCC (hrpG and hpdD half-life ~7.8 min and 6 min, respectively) than in the ΔrsmA mutant cells (hrpG and hpdD half-life ~3.2 min and ~2.8 min, respectively) (Fig. 6B), which likely contributes to the differences in the transcript levels between the ΔrsmA mutant and the wild-type XCC strain observed in the microarray and qRT-PCR experiments (Table 1).

The 5’ UTRs of hrpG and hpdD are 182 nt and 204 nt in length, respectively, which are considered unusually long for bacterial transcripts. RNA structure predictions using Miold [66] suggested that the hrpG and hpdD 5’ UTRs are highly structured, containing loops (Fig. 6C). Additionally, these results suggest that hrpG and hpdD might be targeted by small RNAs in Xanthomonads. The RsmA binding site identified in the 5’ UTR of hrpG was found in a
loop between nucleotides 80 and 120 (Fig. 6C). The predicted structure of hrpD 5' UTR exhibited three GGA motifs between the nucleotides 1 and 120 and within two different loops (Fig. 6D).

RsmA positively regulates HrpG and HrcQ protein levels

The experiments above showed that RsmA can bind and stabilize the hrpG and hrpD transcripts (Figs. 4B and 6A). The first ORF encoded by the hrpD operon (hrqRShtpaA) is hrqQ (Fig. S1), which encodes for an essential C-ring component of the T3SS [80]. Recent findings suggest that HrcQ might act as a general substrate acceptor for T3SS effector proteins due to its interactions with the T3S-ATPase HrcN, the T3S-membrane proteins HrcV and HrcU, and the T3SS effectors [80].

To verify whether the deletion of rsmA in XCC leads to reductions in HrpG and HrcQ protein levels, recombinant constructs encoding HrpG-6His and HrcQ-Flag with native promoters and full-length 5' UTRs were inserted into the chromosomes of wild-type and the ΔrsmA mutant XCC strains using the integrative plasmid pPm7g [81]. The plasmid pPm7g harbors an 800 bp fragment of the α-amylase gene (106–912) of XCC (XAC0798), and its insertion into the XCC genome did not affect pathogenicity [81]. Additionally, ΔrsmA carrying the HrpG-6His construct was transformed with a plasmid-borne pUFRRsmA-Flag to generate a complementation strain. All strains were grown in the XVM2 medium for 20 h, and total proteins were extracted for Western-blotting analysis. The deletion of rsmA resulted in reduced levels of HrpG-6His and HrpQ-Flag to 38% and 32%, respectively, in relation to the wild-type protein levels (Figs. 7A and 7B, respectively). The complemented strain restored the HrpG-6His and HrpQ-Flag levels to that of the wild-type strain (Fig. 7A and 7B). The relative protein values estimated for HrpG-6His and HrpQ-Flag were normalized with the bands detected in the total protein extracts probed with antibodies against the β-subunit of E. coli RNA polymerase (Abcam, ab12087), which was used as a loading control (Figs. 7A and 7B).

Ectopic expression of hrpG in the ΔrsmA mutant restores its virulence and HR-triggering activity

The results presented above suggest that RsmA contributes to the virulence of XCC by stabilizing the transcripts of hrpG, the master regulator of T3SS, and it also has a direct effect on the expression of hrpD operon. To further test our hypothesis that RsmA mostly exerts its regulation of the hrp/hrc genes via hrpG, we ectopically expressed hrpG-6His in the ΔrsmA mutant of XCC. The ectopic expression of hrpG-6His in the XCC ΔrsmA mutant not only restored its virulence and bacterial population levels in the host plant sweet orange, but also contributed to HR elicitation ability in the non-host plant N. benthamiana (Figs. 8A, 8B and 8C). Previously, the XCC wild-type strain 306 was shown to be able to elicit a visible HR in tobacco seven days after inoculation (Fig. 1C).
However, the constitutive expression of *hrpG*-6His in the wild-type strain carrying pBBR5Lac-*hrpG*-6His can trigger a strong HR in tobacco within two days after inoculation (Fig. 8B).

To determine whether the post-translational modification of HrpG is affected by the deletion of *rsmA* in XCC, we generated mutations in the critical residues E44 and D60 within the response regulator domain of the HrpG. The *hrpG*-6His constructs bearing the E44K and D60N mutations were fused to the Lac promoter of the pBBR5 plasmid to obtain recombinant constructs pBBR5Lac-*hrpG*E44K-6His and pBBR5Lac-*hrpG*D60N-6His. It was observed that pBBR5Lac-*hrpG*-6His and pBBR5Lac-*hrpG*E44K-6His recovered the virulence and the ability to elicit HR of the Δ*rsmA* mutant (Figs. 8A, 8B and 8C). The mutant HrpGE44K was previously demonstrated to be constitutively active in *Xanthomonas* by an unknown mechanism that might involve an increase in the affinity between the HrpG response regulator domain and the α-subunit of RNA polymerase [11,82]. On the other hand, Lac-*hrpG*D60N-6His did not restore the virulence or the HR-triggering activity of the Δ*rsmA* mutant (Figs. 8A, 8B and 8C). These findings support previous studies which had suggested that the residue D60 might be essential for phosphorylation and activation of HrpG [11].

Western-blotting experiments confirmed that HrpG-6His, HrpGE44K-6His and HrpGD60N-6His were expressed in the Δ*rsmA* mutant harboring the corresponding pBBR5 constructs, although the Δ*rsmA* mutant contained a lower amount of protein (Fig. 8D).

In vivo phosphorylation of HrpG Asp60 residue is critical to restore the virulence of the Δ*rsmA* mutant, and the deletion of *rsmA* did not affect HrpG phosphorylation

As shown previously, the constitutive expression of *hrpG*-6His completely restored the virulence and HR in the Δ*rsmA* mutant, although the mutated *hrpG*D60N-6His failed to elicit canker symptoms and restore impaired bacterial growth in host plants (Figs. 8A and 8C). We next tested whether the loss in the function of *hrpG*D60N-6His was caused by impaired phosphorylation in the Δ*rsmA* mutant. We utilized the Manganese(II)-Phos-tag (Wako, USA) acrylamide gel system to analyze the in vivo levels of HrpG6His, P. Both HrpG-6His and HrpG-6His-P were detected in the cytoplasm of the XCC wild-type and Δ*rsmA* mutant strains (Fig. 9A). These two HrpG6His isoforms were also present in the mutant ΔH1N and E44K backgrounds, but only the non-

Figure 6. Analysis of *hrpG* and *hrpD* mRNA stability in the wild-type and *rsmA* mutant strains by RT-PCR. A) Cells of the XCC wild-type and Δ*rsmA* strains were grown in XVM2 medium to OD_{600 nm}=0.6, treated with 10 μg/mL ciprofloxacin and harvested at several time points after treatment. Total RNA was isolated, and 2 μg of RNA was used for One Step RT-PCR (Qiagen) in 25 μL reactions. Reactions were subjected to PCR amplification for 26 cycles. Ten microliters of each reaction were resolved on a 1.5% agarose gel. The stability of the *hrpD* transcript was evaluated using primers annealing within the first 5′UTR of *hrpG*. The 16S RNA was analyzed as a control for normalizing the *hrpG* and *hrpD* amplification products. B) The relative values of *hrpG* and *hrpD* mRNA half-lives were estimated by determining the average pixel value of each amplified product and subtracting the background using ImageJ software [68,112]. The mean values were normalized to the corresponding 16S amplification product. Mean values derived from two independent experiments are shown. C) Model for the predicted secondary structure of the *hrpG* and *hrpD* leader sequences obtained with MFold software [66]. The positions of GGA motifs in the structures are indicated with arrows. AUG is shown in an open box. doi:10.1371/journal.ppat.1003945.g006
Discussion

In this study, we demonstrated that rsmA of XCC is critical for activating full virulence in host plants and contributing to the elicitation of HR in non-host plants by positively regulating the expression of the hrp/hrc genes and T3SS effector genes. Microarray and qRT-PCR analyses indicated that the hrp/hrc genes and T3SS effector genes are down-regulated in the rsmA mutant (Tables 1 and S2). Western-blotting assays and GUS assays also showed that RsmA activates the hrp/hrc genes at the transcriptional level (Table 1), and the hrpG and hpdD genes at the post-transcriptional level (Fig. 2). These findings are consistent with the previous transcriptomic analyses of rsmA mutants in X. campestris pv. campestris and X. oryzae pv. oryzae [19,20], indicating that RsmA-mediated activation of hrp/hrc genes and T3SS effector genes is conserved in different species of Xanthomonads.

We further showed that RsmA binds specifically to the 5’ UTRs of the hrpG and hpdD mRNAs. Using gel shift mobility assays, we revealed that RsmA binds to the second GGA motif within a loop between nucleotide 80 and 120. Consistently, mutation or deletion of this GGA motif led to diminution of the binding of the hrpG transcript with RsmA (Fig. 5). Interaction of the hrpG and hpdD 5’ UTRs with RsmA seems to stabilize these transcripts by protecting them from RNA decay in XCC cells (Fig. 6). Likewise, the deletion of the rsmA homolog in E. coli reduced flhDC mRNA stability and flagellum gene expression, resulting in impaired swimming motility [43]. A recent report demonstrated that E. coli CsrA binding activates flhDC expression by inhibiting the 5’ end-dependent RNase E cleavage pathway [44]. Although lower abundance was observed for hrpG and hpdD mRNAs in the XCC ΔrsmA mutant, it remains unclear whether these transcripts are targeted by the 5’ end-dependent RNase E pathway in the absence of RsmA binding, which may lead to down-regulation of hrp gene expression in Xanthomonads.

Studies on the regulation of protein synthesis have shown that mRNA secondary structures present in the 5’ UTR can dramatically influence both mRNA stability and translation initiation [83,84,85]. Therefore, protecting the 5’ UTR of mRNA might avoid endonucleolytic cleavage, a rate-limiting step for mRNA decay [85]. Furthermore, similar to flhDC mRNAs that lack a strong Shine-Dalgarno sequence (GGGUGG) [44], hrpG and hpdD also contain weak Shine-Dalgarno sequences (AGGCCG and AGCUAG, respectively). Thus, the interactions between RsmA and the hrpG and hpdD leader sequences can not only promote mRNA stability, but also increase the translation efficiency of these mRNAs in XCC cells. In fact, the translational fusion of hrpG and hrcQ (the first ORF of the hpdD operon) to the gusA gene driven by a constitutive promoter resulted in reduced β-glucuronidase activity, a rate-limiting step for mRNA decay [43]. A recent report demonstrated that E. coli CsrA binding activates flhDC expression by inhibiting the 5’ end-dependent RNase E cleavage pathway [44]. Although lower abundance was observed for hrpG and hpdD mRNAs in the XCC ΔrsmA mutant, it remains unclear whether these transcripts are targeted by the 5’ end-dependent RNase E pathway in the absence of RsmA binding, which may lead to down-regulation of hrp gene expression in Xanthomonads.

The results presented here support our hypothesis that RsmA exerts its positive regulation on the hrp/hrc genes via hrpG, the master transcriptional regulator of hrp/hrc and T3SS effector genes by controlling the expression of the second transcriptional factor HrpX, which can recognize and directly bind to the PIP-box within the hrp cluster and most T3SS effector genes [9,57]. Of all the hrp/hrc transcripts tested here, RsmA directly interacts with the 5’UTRs of hrpG and hpdD but not with that of hrpB, hpcC and hpcD, all of which carry potential GGA motifs in their leader sequences, or hrpF and hrpX, which do not carry potential GGA motifs in their 5’ UTRs. Although the significance

phosphorylated form was observed for the D60N mutant (Fig. 9A). A small amount of HrpG6His–P was observed in the protein extract of wild-type cells grown in the nutrient broth medium, which represses T3SS gene expression (Fig. 9A), but the major fraction of HrpG-6His was not phosphorylated under this condition. These findings suggest that T3SS activation depends on switching the phosphorylated/non-phosphorylated ratio of HrpG isoforms in Xanthomonas cells. Interestingly, phosphorylated isoforms of HrpG6His were observed in the XCC wild-type and Δisoforms of HrpG6His were observed in the XCC wild-type and T3SS-inducible conditions. Taken together, these results indicate that RsmA-mediated activation of hrp/hrc genes and T3SS effector genes is conserved in different species of Xanthomonads.

Figure 7. Immunoblotting experiments showed reduction of HrpG-6His and HrcQ-Flag protein levels in the rsmA mutant cells of Xanthomonas citri subsp. citri. The wild-type, ΔrsmA and complemented strains of XCC carrying the chromosomally inserted recombinant constructs pPM7-hrpG-6His and pPM7-hrcQ-Flag were grown in XVM2 medium to OD600 nm = 0.6. Total cell extracts were analyzed by SDS-PAGE and immunoblotting using specific antibodies. A) For detection of HrpG-6His, after transferred to membrane protein extracts were incubated with anti-6HisTag antibodies (Medical and Biology Lab, MBL). Also, expression of RsmA-Flag was verified only in protein extracts of complemented strain (pUFrmA) probed with anti-FlagTag antibodies (Sigma). B) For detection of HrcQ-Flag, protein extracts were incubated with anti-FlagTag antibodies (Sigma). doi:10.1371/journal.ppat.1003945.g007
of the direct binding of RsmA to the hrpD operon, which contains the T3SS structural genes hrcQ, hrcR, and hrcS, remains to be determined, this function was not able to explain the overall regulation of RsmA on htp/huc and T3SS effector genes. Instead, htpG as the master regulator of T3SS genes, could link the overall positive regulation of T3SS genes by RsmA. This hypothesis is strengthened by the observation that the constitutive expression of htpG-6His in host plants. Error bars represent standard deviations. D) Western-blotting analysis of HrpG-His protein levels in the wild-type and rsmA mutant strains carrying the htpG (hrpG) or mutated htpG alleles (E44K and D60N) under control of a constitutive promoter. Equal amounts of total cell extracts were analyzed by immunoblotting with anti-6HisTag antibodies (MBL, USA).

doi:10.1371/journal.ppat.1003945.g008

Figure 8. Ectopic expression of htpG under control of a constitutive promoter restores full pathogenicity and HR of the rsmA mutant of Xanthomonas citri subsp. citri. A) XCC strains 306 (WT) and the rsmA mutant carrying the empty plasmid pBBR5 (∆rsmA) or pBBR5 with htpG wild-type (hrpG) or htpG alleles with E44K and D60N mutations were inoculated into A) sweet orange (Citrus sinensis) leaves or B) tobacco leaves by infiltrating bacterial cells at a concentration of 10^8 CFU/ml. Sweet orange leaves inoculated with WT and rsmA mutant strains harboring both htpG and htpG-E44K alleles showed canker symptoms 7 days after inoculation, while a strong HR was observed in tobacco leaves only 2 days after infiltration. However, constitutive expression of the htpGD60N allele was not able to recover the pathogenicity and HR in the rsmA mutant. C) In plant growth curve experiments confirmed that the rsmA mutant transformed with both empty pBBR5 (∆rsmA) and pBBR5lac-htpGD60N-6His (D60N) have impaired growth in host plants. Error bars represent standard deviations. D) Western-blotting analysis of HrpG-His protein levels in the wild-type and rsmA mutant strains carrying the htpG (hrpG) or mutated htpG alleles (E44K and D60N) under control of a constitutive promoter. Equal amounts of total cell extracts were analyzed by immunoblotting with anti-6HisTag antibodies (MBL, USA).

doi:10.1371/journal.ppat.1003945.g008

of the direct binding of RsmA to the hrpD operon, which contains the T3SS structural genes hrcQ, hrcR, and hrcS, remains to be determined, this function was not able to explain the overall regulation of RsmA on htp/huc and T3SS effector genes. Instead, htpG as the master regulator of T3SS genes, could link the overall positive regulation of T3SS genes by RsmA. This hypothesis is strengthened by the observation that the constitutive expression of htpG-6His in the ∆rsmA mutant restored its full virulence in host plants and HR-triggering activity in the non-host plant N. benthamiana.

Our results further indicate that RsmA controls htpG mRNA stability but not the signaling pathway involving the phosphorylation of HrpG protein. The phosphorylation of HrpG is required to activate the expression of htpX and regulate downstream htp/huc and T3SS effector genes [9]. Wild-type HrpG, and the mutants HrpGE44K, and HrpGD41N could all be phosphorylated, while HrpGD60N could not be phosphorylated in the XCC cells grown in the XVM2 medium. Consequently, unlike the phosphorylated wild-type, E44K, and D41N mutants, non-phosphorylated HrpGD60N lost the ability to recover pathogenicity or HR-triggering activity of the ∆rsmA mutant. However, we cannot rule out the possibility that the mutation D60N might reduce HrpG protein stability, resulting in a reduction in virulence and HR-triggering activity. The deletion of rsmA in XCC did not affect the phosphorylation of HrpG under the conditions tested here, suggesting that RsmA can control htpG expression and stability but not the signaling pathway that triggers HrpG phosphorylation.

Interestingly, in Salmonella, the expression of the transcriptional activator of T3SS genes, hilA, is directly controlled by three AraC-
like regulators: HilD, HilC, and RtsA [86]. The CsrA protein binds to and prevents the translation of the hilD mRNA [87]. However, during the infection process, the two-component system BarA/SirA activates the expression of the csrB and csrC small RNAs, which inhibit the action of RsmA/CsrA [86,88]. Likewise, in the phytopathogenic bacteria E. carotovora, RsmA targets and destabilizes the transcripts that encode plant-cell-wall degrading enzymes (PCWDEs), which are crucial virulence factors, and HrpL, an alternative sigma factor that regulates the expression of T3SS genes. The small noncoding RNA rsmB, which is a homolog of E. coli and Salmonella csrB, inhibits RsmA and enables the translation of the PCWDE encoding genes and hrpL [89,90].

Moreover, RsmA plays an important role in the interaction between P. aeruginosa and human airway epithelial cells, as shown by the fact that an rsmA mutant failed to induce actin depolymerization and cytotoxicity in epithelial cells due to the decreased transcription of several regulators of T3SS [91].

In contrast to Enterobacteria, E. carotovora, and Pseudomonas spp., the signaling pathway involving RsmA in Xanthomonads is poorly understood as Xanthomonads lacks the small inhibitory RNA rsmB. Recently, a differential RNA sequencing (dRNA-seq) approach was used to identify eight Xanthomonas small RNAs whose expression levels were controlled by HrpG [18]. However, it remains to be determined whether these small RNAs target and activate hrp genes. Furthermore, Schmidtke et al. identified transcripts with unusually long 5’-UTRs, which could indicate extensive post-transcriptional regulation in Xanthomonads [18]. The RsmA target mRNAs hrpG and hrpD identified here also contain long leader regions with loops, suggesting that the expression of T3SS genes may be under several post-transcriptional controls in Xanthomonads. Recent findings demonstrated that in E. coli, the 5’ UTR of the flagellum master regulator flhDC mRNA is targeted for repression by four small RNAs (ArcZ, OmrA, OmrB and OxyS), and for activation by one small RNA (McaS) [92,93].

RsmA seems to positively regulate critical genes involved in pathogen-host interactions such as hrp/hrc and T3SS effector genes in plant pathogenic bacteria. RsmA also regulates features that are controlled by the quorum-sensing signaling pathway such as extracellular enzymes production, cell motility and biofilm formation in bacteria. Fine-tuning of RsmA activity allows bacteria to overcome a variety of environmental challenges such as host defenses by promptly modifying the mRNA stability and regulating the translation initiation of specific transcripts. Future studies designed to identify new components of RsmA regulatory circuits in Xanthomonads, such as small RNAs, two-component

**Figure 9. In vivo phosphorylation of HrpG Asp60 residue is critical to restore the virulence in the ΔrsmA mutant of XCC.** A) Western-blotting analysis of XCC lysates on a 25 μM Phos-tag acrylamide gel (Wako, USA). Bacteria cells were grown in nutrient broth or XVM2 medium (T3SS inducible medium) to OD_{600 nm} = 0.6, and equal amounts of total cell extracts were analyzed by immunoblotting with anti-6HisTag antibodies (MBL, USA). B) Western-blotting analysis to determine HrpG-His protein levels in XCC cell extracts resolved in a 12% acrylamide gel system without Manganese(II)-Phos-tag. Strains analyzed: WT harboring the wild-type hrpG-6His allele; ΔrsmA carrying the wild-type hrpG-6His allele and the mutated hrpG-6His alleles D41N, E44K and D60N. All hrpG-6His constructs were cloned into the pBBR5 plasmid and placed under the control of a constitutive promoter.

doi:10.1371/journal.ppat.1003945.g009
system proteins, and 5′ end-dependent RNAse E cleavage pathways, will shed light on the signaling network of the HrpG-HrpX-mediated expression of the T3S machinery, and also the overlapping of regulation of the virulence traits driven by the quorum-sensing system in plant pathogenic bacteria.

Materials and Methods

Bacterial strains, plasmids, and growth conditions

The plasmids, oligonucleotides and bacterial strains used in this study are listed in Tables S1 and S4. E. coli cells were grown at 37°C in lysogenic broth (LB) medium (1% (w/v) tryptone, 0.5% (w/v) yeast extract, 1% (w/v) sodium chloride, pH 7.5). The XCC wild-type strain 306 (rifampicin resistant) [46] and the mutant strains were routinely grown in nutrient broth (NB), on nutrient agar (NA) at 28°C. For induction of hbp genes, XCC cells were grown in the minimal medium XVM2 (pH 6.7, 20 mM NaCl, 10 mM (NH₄)₂SO₄, 5 mM MgSO₄, 1 mM CaCl₂, 0.16 mM KH₂PO₄, 0.32 mM K₂HPO₄, 0.01 mM FeSO₄, 10 mM fructose, 10 mM sucrose, 0.03% casamino acid) as previously described [57]. Endoglucanase assays were performed as previously described in [94,95], but with the addition of 0.25% L-arabinose.

Construction of the rsmA deletion mutant and complemented strains of X. citri subsp. citri

DNA manipulations and PCR were performed according to standard procedures [98]. Restriction digestion and DNA ligation were performed in accordance with the manufacturer’s instructions (New England Biolabs, USA). To construct the rsmA deletion mutant, approximately 1 kb of the upstream and downstream regions of the rsmA gene (XAC1743) were amplified by PCR from XCC genomic DNA, and the two fragments were ligated to XCC genomic DNA, and the two fragments were ligated to

Generation of expression plasmids in XCC

To produce the fusion of rsmA to an L-arabinose inducible promoter (BAD) in the plasmid pBBR1-6HisrsmA, pET-6HisrsmA (Table S1) was digested with the NdeI/XhoI restriction enzymes, and the DNA fragment encoding 6HisRsmA was inserted into the NdeI/SalI enzyme sites of pBBA (Table S1). To generate a Flag epitope-tagged RsmA containing its native promoter, a fragment encoding the rsmA gene plus 1 kb upstream sequence was amplified by PCR using the primers F1-rsmAflag and R1-rsmAflag, generating fragment was digested with the BamHI and XhoI restriction enzymes and cloned into the BamHI/XhoI enzyme sites of pUFR047. The resulting construct pUFR-rsmAflag was used to transform the XCC ΔrsmA mutant strain by electroporation. To fuse hrgG to a constitutive promoter, the construct pBBRLac-hrgG6his was produced by PCR amplification of the hrgG orf plus 50 nt upstream using the F2w-hrgG and R-hrgG primers (Table S3). The generated fragment was digested with the PmlI/XhoI restriction enzymes and cloned into the same enzymatic sites of pBBR1-MCS-5. The plasmid pBBR1-MCS-5 contains a lac promoter that promotes constitutive gene expression in Xanthomonas [100]. The construct pBBRLac-hrgG6his was then used as a template to produce HrpG point mutants hrgGD41N-6his, hrgGE44K-6his, and hrgGD60N-6his using the QuikChange Site-Directed Mutagenesis Kit (Stratagene). The constructs hrgG-6his and hrgQ-Flag with their native promoters were produced by two parallel PCR reactions using XCC genomic DNA as the template and the primers F-hrgG/R2-hrgGflag and F-hrcQ/R2-hrcQflag generating the restriction enzyme sites within the amplified fragments (Table S3). The recombinant constructs hrgG-6his and hrgQ-Flag were digested and cloned into the BamHI/XhoI enzyme sites of the pPM7g plasmid producing hrgG6his-pPM7g and hrgQflag-pPM7g (Table S1). These plasmids were used to construct RsmA constructs to insert hrgG6his and hrgQflag into the XCC chromosome. The pPM7g plasmid harbors an 800 bp fragment extracted from the α-amylase gene (106–912) of XCC (XAC0798), and its insertion into XCC chromosome did not affect pathogenicity [81]. XCC cells transformed with pPM7-hrgG-6His and pPM7-hrcQ-Flag constructs were selected and NA with kanamycin and confirmed by PCR. All constructs were confirmed by sequencing.

Pathogenicity and HR assays

Pathogenicity assays were conducted in a quarantine greenhouse facility at the Citrus Research and Education Center, Lake Alfred, FL, U.S.A. Assays were performed using fully expanded, immature leaves of sweet orange (Citrus sinensis), XCC wild-type and mutant strains used in this assay were grown with shaking overnight at 28°C in NB, centrifuged, and suspended in sterile tap water, and the concentrations were adjusted to 10⁶ CFU/ml. For the pathogenicity assays, bacterial solutions of both 10⁶ and 10⁷ CFU/ml were infiltrated into the leaves with needleless syringes [9,101]. Disease symptoms were photographed at 7 days post-inoculation. The strains were also tested for their ability to elicit an HR on N. benthamiana by infiltration of plant tissue with strains adjusted to 10⁶ CFU/ml with a needleless syringe. Plant responses were scored for HR in tobacco 2 days and 7 days post-inoculation. Tobacco plants were grown in growth chambers at 25°C with a 12 h photoperiod. Experiments were repeated three times with similar results.
RNA extraction and qRT-PCR assays

Single bacterial colonies were picked and grown in 5 ml of NB at 28°C for 24 h with shaking, and then transferred into 50 ml of NB for overnight incubation. The bacterial cultures in the middle exponential stage were centrifuged, washed twice with XVM2 medium, and then inoculated in XVM2 medium at an initial concentration of OD_{600 nm} 0.03. Bacteria were grown in XVM2 medium or XVM2 plus 0.25% L-arabinose with shaking at 200 rpm at 28°C, and samples of the cultures were collected at OD_{600 nm} 0.5. Four biological replicates were used for each strain for qRT-PCR and microarray analyses. RNA was stabilized immediately by mixing the bacterial culture with two volumes of RNA protect bacterial reagent (Qiagen), CA, U.S.A.) and incubated at room temperature for 5 min. Bacterial cells were centrifuged at 5000 x g for 10 min and cell pellets were used for RNA extraction. Cell pellets were treated with lysozyme, and RNA extractions were performed using an RNeasy Mini kit (Qiagen). Purified RNA was treated with DNase I-Free (Qiagen), and successful removal of genomic DNA was confirmed by PCR. RNA quantity was initially determined on a ND-8000 Nanodrop spectrophotometer (NanoDrop Technologies, U.S.A.) and RNA quality was assessed using the Agilent 2100 bioanalyzer (Agilent Technologies, CA, U.S.A.). For qRT-PCR assays, reverse transcription was performed using 1 μg of DNaseI-treated RNA and the RevertAid H-Minus First Strand cDNA Synthesis Kit, following the manufacturer’s protocol (Fermentas). Quantitative amplification of the resulting cDNA (40 ng) was performed using 0.3 μM of each primer (Table S4) and SYBR Green/ROX qPCR Master Mix in the ABI7300 Real-Time System (Applied Biosystems). Relative quantification of gene expression was performed using 16S and XAC1631 (which codes for subunit A of DNA gyrase) as endogenous controls and the 2^(-ΔΔCt) method [102]. Primers were designed using the Primer Express Software (Applied Biosystems).

Microarray analyses

For transcriptome studies of the ΔrsmA and ΔrsmA mutant carrying pBRA-ram1, an Agilent 8-by-15K microarray containing 60-mer oligonucleotides for all predicted ORFs in the XCC strain 306 genome were used as described previously [9]. Labeled cDNA was generated using a Fairplay III microarray labeling kit (Agilent Technologies). Total RNA input (5 μg) was used to generate labeled cDNA according to the manufacturer’s protocol. Briefly, cDNA was synthesized from 5 μg of total RNA with AffinityScript HC and random primer, and then modified cDNA was labeled with either cy3 or cy5. The labeled cDNA was purified following the manufacturer’s instructions [103]. The mean signal intensities and statistics of the data were carried out as described previously [9]. Log2-transformed values were used for statistical analysis. A linear modeling approach and the empirical Bayes statistics for differential expression analysis were employed as in the report by [104]. P values were adjusted using the Benjamin and Hochberg method, designated as FDR [105]. Differentially expressed genes were ranked based on FDR, and genes with FDR<0.05 and a minimum absolute value of log2-fold-change > = 1 (equivalent to 2-fold) were considered to be significantly differentially expressed.

Western-blotting and in vivo phosphorylation analyses

To analyze the protein levels in XCC, cells were grown in XVM2 medium to an OD_{600 nm} of 0.5. Bacterial cells were harvested and pellets were resuspended in 50 mM Tris-HCl (pH 8.0), 100 mM NaCl, 0.1% Tween-20, and 1 mg ml ^{-1} lysozyme. Cells were ruptured by sonication. Cell-free extract was prepared by centrifugation at 15, 000 g at 4°C for 45 min. Protein extracts were quantified by the BCA protein assay. Equal amount of proteins were loaded and separated by 15% SDS-PAGE, transferred to a nitrocellulose membrane (GE Healthcare) followed by immunoblotting analysis. Rabbit polyclonal antibodies raised against XCC HrcU, HrpB1, HrpB2, and HrpD6 were previously reported in Cappelletti et al. [57]. The rabbit sera against Hrp proteins were diluted to 1:1000, while anti-Flag (Sigma) and anti-6His (BML, USA) were diluted to 1:5000. Rabbit antibodies were detected with staphylococcal protein-A conjugated to horseradish peroxidase (Sigma). The in vivo levels of phosphorylated HrpG were detected using the Manganese(II)-Phos-tag (Wako, USA) acrylamide gel system. Formic acid-lysed XCC cells grown in XVM2 media to mid-log phase (OD_{600 nm} = 0.6) at 30°C were fractionated on a 12.5% acrylamide gel as described previously [106,107]. After electrophoresis, the gel was washed twice with transfer buffer plus 5 mM EDTA pH 8, and the resolved proteins were then transferred to a nitrocellulose membrane. The membrane was then incubated with anti-6HisTag antibody (BML, USA) followed by protein A-peroxidase. As a control to these experiments, immunoblotting was carried out for the same protein extracts resolved on a 12.5% acrylamide gel without Manganese(II)-Phos-tag. Immunoblots were developed with SuperSignal West Fermo Chemiluminescent Substrate (Pierce) and exposed to CL-XPosure Film (Pierce).

GUS activity assay

To generate the translational constructs with hpg gene-gusA fusion under control of the hpg genes native promoters, the promoter and 5’ UTRs plus three first codons of the hpg genes were fused in frame to the promoterless gusA gene without its ribosome binding site [108]. The upstream sequence and first codons of the hpg genes were amplified by PCR using the total DNA of the XCC wild-type strain 306 as the template. The gusA orf was amplified from the vector pBl121 (Table S1). The primer pairs used in these PCR reactions are listed in Table S3. Fragments containing the translational hpg gene-gusA fusions were first cloned into the pGEM T-easy vector, and then digested with EcoRI/HindIII and inserted into the same enzymatic sites of pLAfR3 [109] (Table S1). Further, the translational constructs with hpg gene-gusA fusion driven by a constitutive promoter were produced after amplifying the leader sequence of hpg genes plus gusA from the constructs described above (Table S3). The PCR products were purified, digested with BamHI/HindIII and inserted downstream of the lac promoter in the vector pUC18-mini-Tn7T-LAC [110]. Those constructs were used to transform XCC wild-type and rsmA mutant strains. Transformed colonies were selected on NA medium plus tetracycline or gentamicin. Wild-type and rsmA mutant cells harboring plasmid-borne promoterless gusA fused in frame to the hpg genes were cultured in XVM2 for 20 h and assayed for β-glucuronidase (GUS) activity. Bacteria cells were diluted and disrupted in a GUS assay buffer (20 mM Tris-HCl, pH 7.0, 10 mM 2-mercaptoethanol, 5 mM EDTA, and 1% TritonX-100). GUS activities were determined at intervals of 15 min for 4 h by measuring the OD_{420 nm} using PNPG.
(p-Nitrophenyl-β-D-glucuronide) as the substrate [60]. The relative GUS activity was expressed as \(1,000 \times A_{420}/\text{time (in min)} \times \text{OD}_{600}\) in Miller units [U] \([27,111]\). Values presented are means ± standard deviations of three independent experiments. The GUS assay was repeated twice with similar results.

5′-RACE

The transcriptional start sites of hpg and hrpX were determined using the 5′/3′ RACE Kit (Roche), according to manufacturer’s instructions. Briefly, total RNA was obtained from XCC wild-type cell cultures grown in XVM2 medium to an OD \(_{600}\) of 0.5. After treatment with DNase I-free (Qiagen), the RNA was reverse transcribed using a gene-specific primer (SP1, Table S4), purified and poly(dA) tailed at the 3′ end by reaction with terminal transferase enzyme. The resulting cDNA was amplified by PCR using the poly-dT primer provided by the kit to anneal at the poly(dA) tail and a gene-specific primer (SP2, Table S3), complementary to a region upstream of the original cDNA primer. The amplicons from the first PCR were submitted to a second PCR reaction using the poly dT primer and a distinct gene-specific nested primer (SP3, Table S3) that was internal to the first PCR products. The PCR products were ligated into the pGEM-T vector (Promega) and several distinct clones were sequenced.

Expression and purification of 6HisRsmA in E. coli

For expression of the recombinant protein 6HisRsmA in E. coli, a fragment containing the rsmA gene sequence was amplified by PCR from XCC genome using primers and F-peTrsmA and R-peTrsmA (Table S4), digested with NotI/EcoRI and inserted into pET-28a (Novagen) resulting in pET-6HisRsmA. Recombinant 6HisRsmA protein was over-expressed in E. coli BL21 Star (DE3) pLysS (Novagen) by addition of 1 mM isopropyl-b-D-thiogalactopyranoside to cells grown to mid-exponential phase. Bacterial cells were harvested 4 h later. Cell pellets were resuspended in 50 mM NaH\(_2\)PO\(_4\) (pH 8.0), 300 mM NaCl, 10 mM imidazole and 1 mg ml\(^{-1}\) lysozyme. Cells were ruptured by sonication. Cell-free extract was prepared by centrifugation at 14,000 g at 4°C for 45 min. 6HisRsmA was purified by Ni-NTA affinity chromatography (Pierce) according to the manufacturer’s recommendations. The protein-containing fractions were concentrated and the buffer was exchanged with 10 mM HEPES pH 7.3, 20 mM KCl, 1 mM MgCl\(_2\), 1 mM DTT, 5% Glycerol, 0.1 μg/μL yeast tRNA, 20 U RNasin (Promega) to a total volume of 20 μL reaction. The binding reactions were incubated at 25°C for 20 min to allow RsmA–RNA complex formation. A total of 5 μL loading buffer (97% glycerol, 0.01% bromophenol blue, 0.01% xylene cyanol) was added to the binding reaction and immediately loaded and separated using 5% native polyacrylamide gels. Signal bands were visualized using the LightShift Chemiluminescent RNA EMSA Kit (Thermo Scientific, USA) according to the manufacturer’s instructions. Their approximately 65 nM 6HisRsmA protein and 6.25 nM Biotin-labeled RNA were mixed into a tube with binding buffer ([10 mM HEPES pH 7.3, 20 mM KCl, 1 mM MgCl\(_2\), 1 mM DTT, 5% Glycerol, 0.1 μg/μL yeast tRNA, 20 U RNasin (Promega)] to a total volume of 20 μL reaction. The binding reactions were incubated at 25°C for 20 min to allow RsmA–RNA complex formation. A total of 5 μL loading buffer (97% glycerol, 0.01% bromophenol blue, 0.01% xylene cyanol) was added to the binding reaction and immediately loaded and separated using 5% native polyacrylamide gels. Signal bands were visualized using the LightShift Chemiluminescent RNA EMSA Kit (Thermo Scientific) according to the manufacturer’s instructions. In the competition assays and for calculating apparent K\(_D\) values of RsmA-hrgG2 complex the concentrations of the 6HisRsmA and RNA probes are indicated in the respective figures and legends. The binding curve for 6HisRsmA-Xcc-hrgG2 interaction was defined as a function of 6HisRsmA concentrations and relative values of shifted band intensity. The apparent equilibrium binding constant (K\(_D\)) was estimated based on the average pixel value of each shifted band calculated with ImageJ software \([68,69,112]\). The calculated mean pixel values (P-value <0.05) were obtained as results of three independent assays.

mRNA stability assay

Bacterial cultures of XCC wild-type and rsmA mutant were grown at 28°C in XVM2 medium to OD\(_{600}\) \(_{\text{mean}}\) of 0.6 and treated with ciprofloxacin at a final concentration of 10 mg/ml to inhibit transcription. Samples were collected at 0, 2, 4, 6, 8 and 15 min after ciprofloxacin treatment. The cells were harvested by centrifugation at 10,000 r.p.m. and used immediately for RNA extraction by addition of Trizol (Life, USA) in the pellets, and heating at 65°C for 10 min. After the separation of organic and aqueous phase, the supernatants containing total RNA were transferred to new eppendorf tubes and RNA extraction was continued by using RNAeasy Mini Kit (Qiagen). Total RNA samples were treated with DNase I-Free (Qiagen) and quantified by using Nanodrop. A total of 2 μg of treated RNA was used for One-Step RT-PCR (Qiagen) in 25 μL reactions following the manufacturer’s protocol. Reactions were subjected to PCR amplification for 26 cycles. Ten microliters of each reaction was resolved in 1.5% agarose gel. Stability of hrpD transcript was evaluated with primers annealing within the first orf hrpD (Table S4). 16S RNA transcript was evaluated with primers annealing within the first orf hrpQ (Table S4). Analysis of 16S RNA transcripts were produced using RT-PCR (Qiagen). The hrp transcripts were produced in vitro by using the T7 Transcription kit (Roche) and Biotin RNA labeling Kit (Roche) following the manufacturer’s instructions. Transcripts were purified by using the Zymoclean Gel RNA Recovery kit (Zymo Research, USA), re-suspended in DEPC water and quantified using Nanodrop. Biotin-labeled RNA probes, in vitro transcribed (Table S3) or synthesized by IDT corporation (Table S4), were heated to 95°C for 3 minutes and reconstituted by incubating 15 minutes at room temperature. The binding assays were performed using the LightShift Chemiluminescent RNA EMSA Kit (Thermo Scientific, USA) according to the manufacturer’s instructions. Their approximately 65 nM 6HisRsmA protein and 6.25 nM Biotin-labeled RNA were mixed into a tube with binding buffer ([10 mM HEPES pH 7.3, 20 mM KCl, 1 mM MgCl\(_2\), 1 mM DTT, 5% Glycerol, 0.1 μg/μL yeast tRNA, 20 U RNasin (Promega)] to a total volume of 20 μL reaction. The binding reactions were incubated at 25°C for 20 min to allow RsmA–RNA complex formation. A total of 5 μL loading buffer (97% glycerol, 0.01% bromophenol blue, 0.01% xylene cyanol) was added to the binding reaction and immediately loaded and separated using 5% native polyacrylamide gels. Signal bands were visualized using the LightShift Chemiluminescent RNA EMSA Kit (Thermo Scientific) according to the manufacturer’s instructions. In the competition assays and for calculating apparent K\(_D\) values of RsmA-hrgG2 complex the concentrations of the 6HisRsmA and RNA probes are indicated in the respective figures and legends. The binding curve for 6HisRsmA-Xcc-hrgG2 interaction was defined as a function of 6HisRsmA concentrations and relative values of shifted band intensity. The apparent equilibrium binding constant (K\(_D\)) was estimated based on the average pixel value of each shifted band calculated with ImageJ software \([68,69,112]\). The calculated mean pixel values (P-value <0.05) were obtained as results of three independent assays.
Mean values derived from two independent experiments are shown.

Supporting Information

Figure S1 Genetic organization of hrp gene clusters of X. citri subsp. citri. The open reading frames which form the transcript units in hrp cluster, hpdA to hpdF, are represented by thick arrows in the map. The PIP boxes positions in each transcript unit and their orientations are indicate by thin arrows, except to hpdA which does not contain a PIP box, orientation of the promoter is shown with dashed arrows. hrc genes encode proteins conserved among type 3 secretion systems; hpd and hpa genes encode non-conserved proteins involved with hypersensitive response and pathogenicity.

Figure S2 Graphical representation of the multiple sequence alignment and structural model of RsmA of Xanthomonas citri subsp. citri. A) Sequences of RsmA homologous proteins were used to generate this representation using the WebLogo server (http://weblogo.berkeley.edu/) [113], in which the height of the residue symbol indicates the degree of conservation and the numbers refer to residue positions in RsmA. RsmA secondary structure was predicted by Phyre (http://www.sbg.bio.ic.ac.uk/~phyre/) [50]. In of the predicted RsmA secondary structure, boxes indicate alpha-helices and arrows indicate beta-strand motifs. B) The RsmA structure model was obtained using the Swiss-Model Repository program with the E. coli CsrA structure as a model [PDB: 1Y00, [48]]. The structure was generated using PyMOL [114]. The critical residues involved in RNA binding are highlighted in A) with stars and in B) with sticks generated using PyMOL [114]. The critical residues involved in RNA binding are highlighted in A) with stars and in B) with sticks.

Figure S3 Induced expression of 6HisRsmA restores the production levels of endoglucanases in rsmA mutant of X. citri subsp. citri. A) Expression of 6HisRsmA upon induction with 0.25% L-arabinose was confirmed by Western-blot analysis with anti-6His antiserum. X. citri subsp. citri strains were growth in XVM2 medium at 28°C and OD600 nm 0.5, and after 4 hours of induction with 0.25% L-arabinose the cells were collect and centrifuged. Supernatants were discarded and cells were resuspended in L-mannil sample buffer. Proteins extracts were analyzed by SDS-PAGE, transferred to nitrocellulose membrane and incubated with anti-6His antiserum. E. coli cell extract expressing the recombinant protein 6HisRsmA was used as a positive control. B) Induced expression of 6HisRsmA restores the production levels of endoglucanases in rsmA mutant of X. citi subsp. citri. Endoglucanases activity assays were performed with supernatant of the strains growth in NB medium supplemented with 0.25% L-arabinose at 28°C and OD600 nm 1.0. Wt, XCC strain 306C; pBRA, mutant rsmA harboring empty plasmid pBRA; pBRA6HisrsmA, rsmA mutant transformed with plasmid-borne 6HisrsmA under control of an arabinose induced promoter.

Table S1 Bacterial strains and plasmids used in this study.

Table S2 Comparison of expression of the genes regulated by rsmA in Xanthomonas citri subsp. citri using microarray.

Table S3 Oligonucleotides primers used in this study.

Table S4 RNA probes encoding 5’-UTR of hrp genes and control used in this study.

Author Contributions

Conceived and designed the experiments: NW MOA CSF. Performed the experiments: MOA. Analyzed the data: MOA NW CSF. Contributed reagents/materials/analysis tools: MOA NW CSF. Wrote the paper: MOA NW CSF.
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