Crp-Like Protein Plays Both Positive and Negative Roles in Regulating the Pathogenicity of Bacterial Pustule Pathogen *Xanthomonas axonopodis* pv. *glycines*

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ABSTRACT

The global regulator Crp-like protein (Clp) is positively involved in the production of virulence factors in some of the *Xanthomonas* spp. However, the functional importance of Clp in *X. axonopodis* pv. *glycines* has not been investigated previously. Here, we showed that deletion of *clp* led to significant reduction in the virulence of *X. axonopodis* pv. *glycines* in soybean, which was highly correlated with the drastic reductions in carbohydrates utilization, extracellular polysaccharide (EPS) production, biofilm formation, cell motility, and synthesis of cell wall degrading enzymes (CWDEs). These significantly impaired properties in the *clp* mutant were completely rescued by a single-copy integration of the wild-type *clp* into the mutant chromosome via homologous recombination. Interestingly, overexpression of *clp* in the wild-type strain resulted in significant increases in cell motility and synthesis of the CWDEs. To our

surprise, significant reductions in carbohydrates utilization, EPS production, biofilm formation, and the protease activity were observed in the wild-type strain overexpressing *clp*, suggesting that Clp also plays a negative role in these properties. Furthermore, quantitative reverse transcription polymerase chain reaction analysis suggested that *clp* was positively regulated by the diffusible signal factor-mediated quorum-sensing system and the HrpG/HrpX cascade. Taken together, our results reveal that Clp functions as both activator and repressor in multiple biological processes in *X. axonopodis* pv. *glycines* that are essential for its full virulence.

Keywords: Clp (Crp-like protein), *Glycine max*, positive and negative regulators, virulence, *Xanthomonas axonopodis* pv. *glycines*

The phytopathogens belonging to the genus *Xanthomonas* cause a variety of diseases in economically important plants worldwide (Büttner and Bonas 2010). These bacteria use quorum sensing (QS) or cell-to-cell communication mediated by diffusible signal factor (DSF) to regulate a diverse range of physiological activities contributing to virulence and adaptation of the pathogens in host plants (He and Zhang 2008; O'Connell et al. 2013; Ryan et al. 2015). DSF signals-mediated QS was firstly discovered and characterized in the black rot pathogen *X. campestris* pv. *campestris* (Barber et al. 1997; Wang et al. 2004). The biosynthesis, perception, and transduction of DSF signals in *Xanthomonas* spp. are executed by the proteins encoded by the regulation of pathogenicity factors (*rpf*) gene cluster. The *rpfF*, encoding an enoyl-coenzyme A hydratase, catalyzes the biosynthesis of DSF, whereas the products of *rpfC* and *rpfG* constitute a two-component regulatory system to sense and transduce the DSF signals (Slater et al. 2000; Ryan et al. 2006; Cai et al. 2017). In addition to RpfC, a novel DSF sensor RpfS, which is a PAS domain-containing histidine kinase, also functions in *X. campestris* pv. *campestris* (An et al. 2014). The DSF sensory system RpfC-RpfG is found in all xanthomonads, whereas

the homologs of RpfS are only present in *X. citri*, *X. axonopodis*, and *X. euvesicatoria* and apparently absent from *Xanthomonas* species attacking monocots, such as *X. oryzae* and *X. translucens* (An et al. 2014; Guo et al. 2012b).

The cyclic dimeric GMP (c-di-GMP) is a conserved second messenger, which presents in all xanthomonads as well as in many other unrelated bacterial pathogens (Nesper et al. 2017; Zhang et al. 2013). The level of c-di-GMP is regulated by at least three categories of proteins containing GGDEF, EAL, or HD-GYP domains (Ryan et al. 2007; Zhou et al. 2013). The GGDEF proteins function as a diguanylate cyclase to synthesize c-di-GMP, whereas the HD-GYP and EAL proteins function as a phosphodiesterase to break the signal molecule c-di-GMP (Zhang et al. 2013; Zhou et al. 2013). RpfG contains a receiver domain for DSF signal transduction and an HD-GYP domain for c-di-GMP degradation (Ryan et al. 2006; Slater et al. 2000). Through controlling the intracellular level of c-di-GMP, the congenital ligand of the global transcription factor Crp-like protein (Clp), RpfG modulates the regulatory activity of Clp (Leduc and Roberts 2009; Tao et al. 2010). In turn, the activated Clp modulates the expression of its target genes either directly by binding to their promoters via the recognition sites TGTGA-N6-TCACA or indirectly through the downstream transcription factor FhrR or Zur (Chin et al. 2010; He et al. 2007). As a result, it regulates diverse biological processes, including synthesis of cell wall degrading enzymes (CWDEs), extracellular polysaccharide (EPS) production, and cell motility, which are essential to the virulence and adaptation of *Xanthomonas* spp. (Guan et al. 2009; He et al. 2007). However, the homologs of FhrR are only present in *X. campestris* pv. *campestris* and apparently absent from the other *Xanthomonas* species, including the parenchyma pathogen *X. axonopodis* pv. *glycines* in soybean.

Genechip analysis revealed that only 94 of 299 genes regulated by Clp are subject to modulation by DSF signals in *X. campestris*

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pv. *campestris* strain XC1 (He et al. 2006, 2007), implying that Clp regulates gene expression not solely through the DSF signaling pathway. Conversely, it is possible that *clp* expression is modulated by additional signaling pathway(s). It has been reported that deletion of *clp* in strain XC1 resulted in a reduced EPS production and decreased activities of both cellulase and protease (He et al. 2007). However, relative to the wild-type strain, an increased protease activity was observed in the *clp* mutant of *X. campestris* pv. *campestris* strain NRRLB1459 (de Crecy-Lagard et al. 1990). Inconsistent with the findings in *X. campestris* pv. *campestris* strain XC1, the Clp homolog in *X. oryzae* pv. *oryzae* strain PXO99^A is not essential for DSF-mediated cellulase production (Guan et al. 2009). All of these findings indicate that different Clp homologs likely possess divergent functions among *Xanthomonas* spp. Although the global regulator Clp is well characterized in the vascular pathogens, such as *X. campestris* pv. *campestris* and *X. oryzae* pv. *oryzae*, the functions of Clp in the parenchyma pathogens, including *X. axonopodis* pv. *glycines*, are not well understood. Interestingly, our data showed that, compared with the *pgk* (encoding a phosphoglycerate kinase) mutant, overexpressing *clp* in the *pgk* mutant significantly reduced its ability in utilization of carbohydrates (unpublished data), implying that Clp of *X. axonopodis* pv. *glycines* is involved in carbohydrates utilization, a property that has not been reported for any of Clp homologs previously.

The Gram-negative bacterium *X. axonopodis* pv. *glycines* is the causal agent of bacterial pustule (BP), one of the most destructive diseases of soybean in several major soybean-producing countries (Athinuwat et al. 2009; Thowthampitak et al. 2008). The pathogen infects soybean leaves through stomata or wound sites and colonizes intercellular spaces in the mesophyll cells, resulting in small pale green spots with raised centers that develop into erumpent pustules (Chatnaparat et al. 2012). The disease progression caused by *X. axonopodis* pv. *glycines* infection is quite distinct from that of *X. oryzae* pv. *oryzae* and *X. campestris* pv. *campestris*, which invade the vascular system of their host plants. Although *X. axonopodis* pv. *glycines* seems to harbor some regulators of virulence factor production in common with *X. oryzae* pv. *oryzae* and *X. campestris* pv. *campestris*, such as the global regulator Clp, it seems likely that its distinct interactions with soybean would necessitate more diverse functions for Clp than those found in the vascular pathogens. Thus, in this study, we investigated the functional roles of Clp in regulating various biological processes to better understand its contributions to the pathogenicity of *X. axonopodis* pv. *glycines*. We provided evidence that Clp functions as both activator and repressor in multiple biological properties, which contribute to the full virulence and adaptation of this pathogen. Our data together with the results from other studies enable us to propose a working model to depict how Clp coordinates the DSF-mediated signals and the HrpG/HrpX signaling cascade that ultimately leads to the full virulence and adaptation of *X. axonopodis* pv. *glycines*.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains and plasmids used in this study are listed in Supplementary Table S1. *Escherichia coli* strains were routinely grown in LB (5 g liter⁻¹ of yeast extract, 10 g liter⁻¹ of tryptone, and 10 g liter⁻¹ of NaCl) medium at 37°C. All *X. axonopodis* pv. *glycines* strains were grown at 28°C in NYG (3 g liter⁻¹ of yeast extract, 5 g liter⁻¹ of polypeptone, and 20 g liter⁻¹ of glycerol), NYG without glycerol (NY), NAN (1 g liter⁻¹ of yeast extract, 3 g liter⁻¹ of beef extract, and 5 g liter⁻¹ of polypeptone), NAS (NAN with 100 g liter⁻¹ of sucrose), NCM [2 g liter⁻¹ of (NH₄)₂SO₄, 4 g liter⁻¹ of K₂HPO₄, 6 g liter⁻¹ of KH₂PO₄, and 0.2 g liter⁻¹ of MgSO₄·7H₂O], or L medium (10 g liter⁻¹ of tryptone, 5 g liter⁻¹ of yeast extract, 5 g liter⁻¹ of NaCl, and 1 g liter⁻¹ of glucose) when required (Guo et al. 2017).

Different antibiotics were added at the following final concentrations as required: carbenicillin, 60 µg ml⁻¹; ampicillin, 60 µg ml⁻¹; kanamycin, 25 µg ml⁻¹; and spectinomycin, 100 µg ml⁻¹.

Constructions of the *clp* deletion mutant, complementation, and *clp*-overexpressing strains. The in-frame deletion mutation of *clp* in *X. axonopodis* pv. *glycines* was generated following the method previously described (Jiang et al. 2009) using the primers listed in Supplementary Table S2. The obtained mutant strain was named NΔ*clp*.

For complementation and overexpressing strains, the entire *clp* (including the promoter and coding regions) was amplified by polymerase chain reaction (PCR), and it was then cloned into pK18mobGII and pHM1 vectors, respectively. The resulting construction pK*clp* and pC*clp* plasmids were then transferred into the mutant strain NΔ*clp* and the wild-type strain, respectively, via electrotransformation (Li et al. 2011), resulting in the complemented strain in the mutant background named CNΔ*clp* and the *clp*-overexpressing strain in the wild-type background named *X. axonopodis* pv. *glycines* (*clp*).

Assays of carbohydrates utilization and EPS production. Qualitative/quantitative analysis of carbohydrates utilization was measured as previously described by Guo et al. (2015). EPS production assays were carried out according to the method described previously by Guo et al. (2017). Each experiment was repeated at least three times, and the data shown are the means of three replicates with standard deviation.

Determination of biofilm formation, cell motility, and exoenzyme activity. Biofilm assays were performed as described previously by Zhang et al. (2013). Swimming motility of *X. axonopodis* pv. *glycines* strains was investigated on semisolid medium plates with 0.3% agar as described by Guo et al. (2017). The exoenzyme activity in culture supernatants of *X. axonopodis* pv. *glycines* strains was analyzed according to the method described previously by Thowthampitak et al. (2008). The experiment was repeated independently three times, and the data presented are from a representative experiment.

Determination of ATP levels. Intracellular ATP levels were determined by using the ATP Assay Kit (Beyotime). Briefly, *X. axonopodis* pv. *glycines* strains were cultured in NY medium supplemented with 2% glucose for 12 or 24 h, and the cells were subsequently harvested and immediately resuspended in ATP detection lysis buffer followed by collection of the supernatant and cooling in an ice bath. Measurements were done by addition of 100 µl ATP detection working buffer to a 20-µl sample. All samples were measured at least in triplicate. Standard ATP curves were generated with standards containing known amounts of ATP in buffer.

Quantitative reverse transcription polymerase chain reaction. Quantitative reverse transcription polymerase chain reactions (qRT-PCRs) were performed using the primers. Briefly, the fresh *X. axonopodis* pv. *glycines* strains were incubated in NYG medium until the OD₆₀₀ value reached 2.0; then, the bacterial cells were collected and washed twice with sterile water. Total RNAs were extracted using the Trizol reagent (Takara) according to the manufacturer's protocol; then, RNA samples were analyzed for quality using gel electrophoresis, and the potential traces of genomic DNA were removed using RNase-free DNase I (TaKaRa). Complementary DNA synthesis was conducted with AMV random primers provided by the manufacturer. qRT-PCRs were performed on the Applied Biosystems 7500 real-time PCR System using SYBR Premix ExTaq (Takara), and the PCR thermal cycle conditions were as follows: denature at 95°C for 30 s and 41 cycles at 95°C for 5 s and 60°C for 34 s. Transcription of the integration host factor A (Thowthampitak et al. 2008) was used as an internal standard to estimate template concentrations. All qRT-PCRs were performed in two independent experiments with four replicates in each test.

Plant assays. Assays for the virulence were performed as described previously (Chatnaparat et al. 2012). Briefly, *Xanthomonas* cells were grown in NYG medium at 28°C with constant shaking at 200 rpm until the exponential phase of growth. Bacterial cells were harvested by centrifugation, washed twice, and resuspended in 1 mM potassium phosphate buffer to OD₆₀₀ ≈ 0.2. Bacteria were then inoculated onto soybean leaves (cultivar Suinong10; susceptible to BP, 6 weeks old) with high-pressure spray for virulence assessment. All plants were grown and maintained in a growth chamber as previously described (Chatnaparat et al. 2012). Plant phenotypes were scored 7 to 10 days postinoculation for pustule symptoms in soybean. Five plants were inoculated for each independent experiment, and each treatment was repeated at least three times.

Bacterial growth within soybean leaves was assessed as previously described by Guo et al. (2017). Briefly, bacterial cell suspensions (OD₆₀₀ ≈ 0.1) were infiltrated into the intercellular spaces of soybean leaves with needleless syringes. Leaves were collected daily for 7 days after infiltration and homogenized in 1 ml of distilled water. Diluted homogenates were plated on NYG agar plates supplemented with appropriate antibiotics. The number of bacterial colonies was counted after incubation at 28°C until single colonies could be counted after 2 to 3 days. The experiments were repeated at least three times.

RESULTS

***X. axonopodis* pv. *glycines* Clp description and generation of the *clp* deletion mutant.** Bioinformatic analysis of the *X. axonopodis* pv. *glycines* genome revealed the presence of a transcriptional regulator Crp homolog that is referred to as Clp hereafter. Clp belongs to Crp superfamily, and it is a protein of 230 amino acids with a predicted molecular mass of 25.6 kDa. Analysis of Clp with the Motif Scan Tool revealed that it contains a cNMP binding domain located in residues 22 to 127 and a Crp-type HTH domain from residues 158 to 230. Clp shares 94 to 99% identity with the CAP-like protein from *X. axonopodis* pv. *citri* strain 306 and *X. campestris* pv. *vesicatoria* strain 85-10 as well as a cAMP-regulatory protein in *X. campestris* pv. *campestris* strain ATCC33913, *X. oryzae* pv. *oryzae* strain PXO99A, and *X. oryzae* pv. *oryzicola* strain BLS256. Using the Neural Network Promoter Prediction Website, the promoter sequence of *clp* was mapped at -79 to -128 nucleotides (with a score of 0.95) upstream of the start codon ATG.

To understand the functional roles of *clp*, the *clp* deletion mutant (named NΔ*clp*) was constructed by homologous suicide plasmid integration (Supplementary Fig. S1). As expected, a 1,533-bp fragment was amplified from the wild type with the primer pair 1F/2R, whereas only a 840-bp DNA fragment was amplified in the *clp* mutant because of an in-frame deletion of 693 bp from *clp*. The in-frame deletion was further verified by a nested PCR using the primer pair 3F/3R. Meanwhile, the complemented and *clp*-overexpressing strains were also constructed by introducing the recombinant plasmids pKClp and pCclp into NΔ*clp* and the wild type, respectively. The authenticities of these two strains were verified by antibiotic screening and PCR, respectively (Supplementary Fig. S2). The resulting complemented strain in the *clp* mutant background was designated as CNΔ*clp*, and the *clp*-overexpressing strain in the wild-type background was designated as *X. axonopodis* pv. *glycines* (*clp*). Whereas only one copy of *clp* was expressed in the complementation strain CNΔ*clp*, multiple copies of *clp* were expressed in the *clp*-overexpressing strain *X. axonopodis* pv. *glycines* (*clp*).

The optimal level of Clp is required for carbohydrates utilization of *X. axonopodis* pv. *glycines*. In the presence of various carbohydrates, we found that overexpressing *clp* in the *pgk* mutant significantly reduced the growth rate compared with that of the *pgk* mutant (unpublished data), implying that Clp might be

involved in carbohydrate utilization of *X. axonopodis* pv. *glycines*. To explore whether mutation in *clp* affects carbohydrates utilization of *X. axonopodis* pv. *glycines*, the growths of NΔ*clp*, CNΔ*clp*, *X. axonopodis* pv. *glycines* (*clp*), and the wild-type strain were evaluated both qualitatively and quantitatively by the established methods (Guo et al. 2012a). The fact that the *clp* mutant grew similarly as well as the wild-type strain in NY medium (without carbohydrate) indicated that the *clp* mutant was not auxotrophic (Supplementary Fig. S3). However, on the NCM medium plates supplied with various sugars or pyruvate individually as the sole carbon source, the *clp* mutant strain grew significantly slower than the wild-type strain. The complemented strain CNΔ*clp* formed similar colonies in sizes to those formed by the wild-type strain. Unexpectedly, instead of forming a larger colony, the *clp*-overexpressing strain *X. axonopodis* pv. *glycines* (*clp*) formed colonies significantly smaller in size compared with the ones formed by the wild-type strain, indicating that Clp is involved in regulating carbohydrates utilization in *X. axonopodis* pv. *glycines*.

To further confirm this result, the carbohydrates utilization of the four tested strains was measured quantitatively by growing them in liquid NCM medium in the presence of various carbohydrates followed by OD₆₀₀ reading at different time points postinoculation. As shown in Figure 1, the growth rates reflected by the reading of OD₆₀₀ were perfectly consistent with the colony sizes shown in Supplementary Figure S4, indicating that the tightly regulated optimal level of Clp is essential for *X. axonopodis* pv. *glycines* in efficiently utilizing carbohydrates. To investigate the effects of supplied carbohydrates on the expression of *clp*, qRT-PCRs were performed to monitor its expression profiles. We found that the expression of *clp* was strongly inhibited by various sugars and pyruvate (Supplementary Fig. S5), suggesting that the expression of *clp* is negatively regulated by these carbohydrates.

Either knockout or overexpression of *clp* reduces EPS production in *X. axonopodis* pv. *glycines*. It has been reported that Clp is required for EPS production in *X. campestris* pv. *campestris* and *X. oryzae* pv. *oryzae* (Guan et al. 2009; He et al. 2007). To explore whether Clp regulates the EPS production in *X. axonopodis* pv. *glycines*, EPS yields were measured both qualitatively and quantitatively. As shown in Supplementary Figure S6, on the NY medium plates with 2% glucose, sucrose, fructose, mannose, galactose, or pyruvate, the *clp* mutant and the *clp*-overexpressing strain *X. axonopodis* pv. *glycines* (*clp*) all formed significantly smaller colonies than the wild-type strain, suggesting that Clp is involved in regulating the EPS production of *X. axonopodis* pv. *glycines*.

To quantitatively evaluate the contribution of Clp to EPS production, all tested strains were cultured in NY medium supplemented with 2% carbohydrates for 5 days, and EPS was subsequently extracted from the cultures and quantified as described here. The results showed that the *clp* mutant produced ~70 to 90% less EPS than the wild-type strain when cultured in the NY medium supplemented with glucose, sucrose, fructose, mannose, galactose, or pyruvate (Fig. 2A). The complemented strain CNΔ*clp* could completely restore the EPS yield to the wild-type level. Interestingly, the *clp*-overexpressing strain *X. axonopodis* pv. *glycines* (*clp*) displayed significantly reduced EPS production compared with that of the wild-type strain (Fig. 2A). These results indicate that either reduced or increased Clp level results in significantly reduced EPS yields, and the optimal level of Clp is essential for EPS production in *X. axonopodis* pv. *glycines*.

The EPS biosynthesis in *Xanthomonas* spp. is mainly controlled by the *gum* and *xan* gene clusters (Guo et al. 2017; Vojnov et al. 2001; Yang et al. 2016). To further understand how Clp regulates EPS biosynthesis in *X. axonopodis* pv. *glycines*, qRT-PCRs were performed to monitor the expression profiles of the genes in *gum* and *xan* clusters. As shown in Figure 2B, the transcriptional levels of the 12 genes in the *gum* cluster were all significantly decreased in the *clp* mutant compared with that in the wild-type strain, indicating that Clp plays a positive role in regulating the expression of *gum*

genes. Additionally, the expressions of *gumB* and *gumC* were significantly lower in *X. axonopodis* pv. *glycines* (*clp*) than that in the wild-type strain (Fig. 2B), further confirming that overexpression of *clp* also negatively regulates the expression of *gumB* and *gumC*. However, no effect of Clp on the expression of the *xan* genes was observed (Supplementary Fig. S7). These results suggest that the *gum* cluster, especially *gumB* and *gumC*, plays a critical role in EPS production of *X. axonopodis* pv. *glycines*.

The optimal level of Clp is critical for the biofilm formation in *X. axonopodis* pv. *glycines*. Biofilm is essential for bacterial virulence, and its formation requires the EPS xanthan (Danhorn and Fuqua 2007; Dow et al. 2003). To investigate whether Clp plays a role in biofilm formation in *X. axonopodis* pv. *glycines*, a biofilm formation assay was performed using a well-established method (Zhang et al. 2013). The biofilm formation was significantly reduced in the *clp* mutant strain compared with that of the

wild-type strain (Fig. 3). The complemented strain CN Δ *clp* could completely restore the biofilm formation to the wild-type level (Fig. 3). However, instead of complementing the biofilm formation to a higher than the wild-type level, overexpression of *clp* in the wild-type strain resulted in a comparable but reduced biofilm formation relative to the wild-type strain (Fig. 3), suggesting that an optimal Clp level is important for biofilm formation and that either an increased or decreased Clp level leads to compromised formation of biofilm in *X. axonopodis* pv. *glycines*.

Clp positively regulates cell motility of *X. axonopodis* pv. *glycines*. To explore whether mutation in *clp* affects cell motility of *X. axonopodis* pv. *glycines*, N Δ *clp*, CN Δ *clp*, *X. axonopodis* pv. *glycines* (*clp*), and the wild-type strain were inoculated onto the tested plates for motility assay. As shown in Figure 4, all tested strains exhibited a comparable motility on the NY plates (Fig. 4A and B), whereas the *clp* mutant displayed a severely compromised

Fig. 1. The optimal level of Crp-like protein (Clp) is required for carbohydrates utilization (A, glucose, B, sucrose, C, fructose, D, mannose, E, galactose, and F, pyruvate) of *Xanthomonas axonopodis* pv. *glycines* (Xag). Aliquots were taken in triplicate at intervals of 144 h after incubation at 28°C, and bacterial growth was measured by OD₆₀₀ readings. Data are means $\pm$ standard deviations from three biological replicates.

cell motility compared with the wild-type strain on NY containing 0.5% glucose plates (Fig. 4A and B). As expected, the defective motility of the *clp* mutant could be completely restored by the complementary plasmid pKC*clp* to the wild-type level (Fig. 4A and B). The *clp*-overexpressing strain *X. axonopodis* pv. *glycines* (*clp*) showed cell motility to a level that higher than that of the wild-type strain (Fig. 4A and B). These results suggested that Clp plays a positive role in regulating cell motility of *X. axonopodis* pv. *glycines*.

To further understand how Clp regulates cell motility in *X. axonopodis* pv. *glycines*, qRT-PCRs were performed to monitor the expression profiles of five genes that have been shown to be vital for cell motility (Tian et al. 2015). The expressions of *fliA* (encoding an RNA polymerase sigma factor for flagellar), *fleN* (encoding a flagellar synthesis regulator), *flhF* (encoding a flagellar biosynthesis protein), *rpoN2* (encoding an RNA polymerase sigma-54 factor), and *fleQ* (encoding a flagellar regulatory protein) were all significantly decreased in the *clp* mutant (Fig. 4C), indicating that the downregulation of these five genes was at least partly responsible for the reduced motility of the *clp* mutant. As expected, the transcriptions of these five genes, especially *fliA* and *fleN*, were significantly increased in the *clp*-overexpressing strain *X. axonopodis* pv. *glycines* (*clp*) (Fig. 4C). Together, these results suggest that Clp positively regulates cell motility, likely through regulating the expression of *fliA*, *fleN*, *flhF*, *rpoN2*, and *fleQ* genes.

ATP provides the energy that drives the swinging of bacterial flagellum, and thus, it is important for bacterial motility (Lu et al. 2009a). To examine the affect of *clp* deletion on the intracellular ATP levels in *X. axonopodis* pv. *glycines*, we quantitated the ATP levels in both the wild-type and the *clp* mutant strains using the ATP Assay Kit (Beyotime). The ATP levels of the *clp* mutant strain were

~80.7 and ~76.9% of that of the wild-type strain when cultured in NY medium supplemented with 2% glucose for 12 and 24 h, respectively, and the intracellular ATP level in the complemented strain was almost restored to the wild-type level (Fig. 4D), indicating that *clp* is required for the accumulation of intracellular ATP in *X. axonopodis* pv. *glycines*. Interestingly, instead of significantly increasing the intracellular ATP level, overexpressing *clp* in the wild-type strain slightly decreased the ATP levels (Fig. 4D), indicating that the optimal expression level of *clp* is required for maintaining the appropriate intracellular ATP level.

Either deletion or overexpression of *clp* reduces protease activity in *X. axonopodis* pv. *glycines*. It has been known that Clp possesses divergent functions on the protease activity of both strains XC1 and NRRLB1459 of *X. campestris* pv. *campestris* (Büttner and Bonas 2010; He et al. 2007). To explore whether deletion of *clp* affects protease activity in *X. axonopodis* pv. *glycines*, the protease activity was determined in the wild-type, *NΔclp*, *CNΔclp*, and *X. axonopodis* pv. *glycines* (*clp*) strains. The protease activity is reflected by the size of the hydrolysis circle. As

Fig. 2. A, Either knockout or overexpression of Crp-like protein (*clp*) reduces extracellular polysaccharide (EPS) production in *Xanthomonas axonopodis* pv. *glycines*. All *X. axonopodis* pv. *glycines* strains were cultured in NY medium supplemented with 2% different carbohydrates for 5 days. The culture supernatants were collected for EPS production assays as described in the text. **Significant differences at $P = 0.01$ by the Student's t test. B, The expression levels of 12 *gum* genes were determined by quantitative reverse transcription polymerase chain reactions. Integration host factor A was used as a reference gene for normalization. Data are means $\pm$ standard deviations from three biological replicates. Xag, *X. axonopodis* pv. *glycines*.

Fig. 3. The optimal level of Crp-like protein (Clp) is critical for the biofilm formation in *Xanthomonas axonopodis* pv. *glycines*. A, The glass-bound biofilm was stained with 0.1% crystal violet. B, The biofilm is quantified as A_{600}/OD_{600} . Data are means $\pm$ standard deviations from three replicates. The different letters above horizontal columns represent significant differences at $P = 0.01$ by the Student's t test. Xag, *X. axonopodis* pv. *glycines*.

expected, deletion of *clp* significantly compromised the protease activity (Fig. 5A and B). The reduced protease activity in the *clp* mutant was completely restored to the wild-type level by the complementary plasmid pKCclp (Fig. 5A and B). Interestingly, compared with that of the wild-type strain, the protease activity was also significantly reduced in the *X. axonopodis* pv. *glycines* (*clp*) strain overexpressing *clp* (Fig. 5A and B). These results indicated that Clp plays both positive and negative roles in regulating protease activity of *X. axonopodis* pv. *glycines*.

To further understand how Clp regulates the protease activities in *X. axonopodis* pv. *glycines*, qRT-PCRs were performed to monitor the expression profiles of 12 protease-related genes (Supplementary Table S3). As shown in Figure 5, the expression levels of *pro1-12* in *NΔclp* were all significantly reduced relative to the wild-type strain, indicating that the *clp* mutant universally represses the expression of *pro1-12*, which at least partially explains the reduced protease activities in *NΔclp*. The most significant reductions in the expression of *pro1*, *pro2*, *pro3*, and *pro11* in the *clp* mutant suggest that these four genes likely contribute the most to the reduction of protease activities observed in the *clp* mutant (Fig. 5C). Although the expression levels of *pro1-4*, *pro8*, *pro10*, and *pro12* were significantly lower in *X. axonopodis* pv. *glycines* (*clp*) than in the wild-type strain, the expression levels of *pro5*, *pro6*, *pro7*, *pro9*, and *pro11* were significantly higher in *X. axonopodis* pv. *glycines* (*clp*) than in the wild-type strain, indicating that overexpression of *clp* plays opposite roles in regulating the expression of these two groups of genes (Fig. 5C).

Clp positively regulates synthesis of α -amylase, endo- β -mannanase, and carboxymethylcellulase in *X. axonopodis* pv. *glycines*. It has been shown that CWDEs contribute to disease symptomatology by facilitating bacterial infection in *Xanthomonas*

spp. (Thowthampitak et al. 2008; Zou et al. 2012). To explore whether deletion of *clp* also affects synthesis of the other exoenzymes in *X. axonopodis* pv. *glycines*, the activities of α -amylase, endo- β -mannanase, and carboxymethylcellulase were measured. Compared with the wild-type strain, synthesis of α -amylase, endo- β -mannanase, and carboxymethylcellulase in the *clp* mutant strain was significantly decreased (Fig. 6A). As expected, the impaired exoenzymes activities in the *clp* mutant could be completely restored by the complementary plasmid pKCclp to the wild-type level (Fig. 6A). Overexpression of *clp* in the wild-type strain resulted in significantly increased activities of these exoenzymes (Fig. 6A). These results indicate that Clp positively regulates synthesis of α -amylase, endo- β -mannanase, and carboxymethylcellulase in *X. axonopodis* pv. *glycines*.

To further understand how Clp regulates the activities of these exoenzymes in *X. axonopodis* pv. *glycines*, qRT-PCRs were performed to monitor the expression of the biosynthesis-related genes for these enzymes (*amyl1* and *amyl2* for α -amylase; *manA* for endo- β -mannanase; and *egl-1*, *egl-2*, and *engXCA* for carboxymethylcellulase). Our results showed that the expression levels of these genes were highly correlated with the enzyme activities displayed in the wild type and the *clp* mutant strain as well as the complemented strain *CNΔclp* and *clp*-overexpressing strain *X. axonopodis* pv. *glycines* (*clp*) (Fig. 6B and C), indicating that Clp plays positive roles in regulating the expressions of the biosynthesis-related genes for these exoenzymes in *X. axonopodis* pv. *glycines*.

***clp* is positively regulated by the DSF-mediated QS system and the HrpG/HrpX cascade.** RpfF, RpfC, and RpfG positively regulate the expression of *clp* in *X. campestris* pv. *campestris* (He et al. 2007), and HrpG indirectly regulates the messenger RNA level of *clp* via GGDEF domain-containing proteins and c-di-GMP in *X. axonopodis* pv. *citri* (Guo et al.

Fig. 4. Crp-like protein (*clp*) is required for cell motility of *Xanthomonas axonopodis* pv. *glycines*. **A**, Cell motility was determined on NY medium plates and NY medium plates supplemented with 0.5% glucose, which is an inducer for motility of *Xanthomonas* spp. **B**, Swimming diameter on plates was measured. Data are means $\pm$ standard deviations from three replicates. The different letters above horizontal columns represent significant differences at $P = 0.01$ by the Student's *t* test. **C**, The transcriptional levels of the flagellar-associated genes were examined by quantitative reverse transcription polymerase chain reactions. **D**, Intracellular ATP levels were determined by using the ATP Assay Kit. The asterisks in each horizontal column result from a paired, two-tailed Student's *t* test relative to the wild type. *Xag*, *X. axonopodis* pv. *glycines*. ** $P = 0.01$; * $P = 0.05$.

2011). To explore whether *clp* expression is regulated by the DSF-mediated QS system and the HrpG/HrpX cascade in *X. axonopodis* pv. *glycines*, qRT-PCRs were performed to examine its expression in different mutant strains. As shown in Figure 7A, the expression of *clp* was significantly downregulated in the *rpfF*, *rpfC*, *rpfG*, *rpfS* (the second sensor for DSF signals), and *rpfR* (encoding a putative

RpfS interplay protein) mutants (An et al. 2014), respectively. In addition, the expression of *clp* was also significantly reduced in *ravS* and *ravR* mutants (both RavS and RavR are involved in the c-di-GMP signaling pathway) (Fig. 7A). These results indicated that the DSF-mediated QS system positively regulates the expression of *clp*. Mutations in *trh*, *xopL* (also called *lrpX*) (Islam et al. 2009), *zur*,

Fig. 5. Either knockout or overexpression of Crp-like protein (*clp*) reduces protease activity in *Xanthomonas axonopodis* pv. *glycines*. **A**, Protease activity was determined on 1% milk plates and photographed after 2 to 3 days of incubation at 28°C. The assays were done three times, each with four replicates, and a representative plate for each assay is shown. **B**, The protease activities are reflected by the diameters of hydrolysis circle on plates. Data are means $\pm$ standard deviations from three replicates. The different letters above horizontal columns represent significant differences at $P = 0.01$ by the Student's *t* test. **C**, The transcriptional levels of the 12 protease genes were determined by quantitative reverse transcription polymerase chain reactions. *Xag*, *X. axonopodis* pv. *glycines*.

rsmA (a positive regulator of *hrpG*) (Andrade et al. 2014), *hrpX*, and *hrpG*, which are components of the HrpG/HrpX cascade, also reduced the expression of *clp*, indicating that the HrpX/HrpG cascade positively regulates the *clp* expression (Fig. 7B).

***clp* contributes to the full virulence of *X. axonopodis* pv. *glycines* in soybean.** Because disruption of *clp* significantly impaired the multiple biological properties of *X. axonopodis* pv. *glycines*, it prompted us to investigate whether Clp contributes to the virulence of *X. axonopodis* pv. *glycines*. As shown in Figure 8A, the *clp* mutant exhibited significantly reduced disease symptoms in soybean compared with those caused by the wild-type strain. The reduced disease symptom caused by the Δclp strain could be

completely complemented by the complementary plasmid pKC*clp* (Fig. 8A). Interestingly, instead of increasing the disease symptom, overexpressing *clp* in the wild-type strain resulted in significant reductions in symptom severity relative to the wild-type strain (Fig. 8A). As a negative control, the T3SS deletion mutant $\Delta hrcC$ did not cause any severe symptom in soybean (Fig. 8A). The disease symptom is usually affected by many factors, and thus, is not an accurate indicator for bacterial virulence. To accurately evaluate the effects of *clp* deletion and overexpression on the virulence of *X. axonopodis* pv. *glycines*, the bacterial growth assays were performed in soybean leaves for 7 days. As shown in Figure 8B, the bacterial growth rates at different time points were highly correlated with the

Fig. 6. Crp-like protein (Clp) positively regulates the synthesis of α -amylase, endo- β -mannanase, and carboxymethylcellulase activity in *Xanthomonas axonopodis* pv. *glycines*. **A**, The activities of **a**, α -amylase, **b**, endo- β -mannanase, and **c**, carboxymethylcellulase were detected on plates and photographed after 2 to 3 days of incubation at 28°C. The activity assays were done three times, each with four repeats, and a representative plate for each assay is shown. **B**, The transcriptional levels of α -amylase- and endo- β -mannanase-related genes were determined by quantitative reverse transcription polymerase chain reactions (qRT-PCRs). Data are means $\pm$ standard deviations from three replicates. **C**, The transcriptional levels of the carboxymethylcellulase-associated genes were determined by qRT-PCRs. *Xag*, *X. axonopodis* pv. *glycines*.

symptom severities for the tested strains, indicating that an optimal level of Clp is required for the full virulence of *X. axonopodis* pv. *glycines*. Either reduced or enhanced level of Clp has a negative impact on the full virulence of *X. axonopodis* pv. *glycines*.

DISCUSSION

In *Xanthomonas* spp., DSF-mediated QS or cell-to-cell communication plays an essential role in signal perception and signal transduction for coordinating the production of a range of virulence factors and biofilm dispersal. Clp, as a key component in DSF-dependent signaling regulation, was first identified in the vascular pathogen *X. campestris* pv. *campestris* and found to positively regulate EPS production, cell motility, and cellulase and protease activities but not regulate biofilm dispersal (He et al. 2007). Clp was further reported to positively regulate EPS production and cell motility but not regulate biofilm formation and cellulase activity in the other vascular pathogen *X. oryzae* pv. *oryzae* (Guan et al. 2009). Furthermore, Clp was shown to negatively regulate protease activity in another strain of *X. campestris* pv. *campestris* (de Crecy-Lagard et al. 1990). In this study, we provided evidence that the global regulator Clp was positively regulated by the DSF-mediated QS system and the HrpG/HrpX cascade and that it functions as both activator and/or repressor in carbohydrates utilization, EPS production, biofilm formation, cell motility, and synthesis of CWDEs of *X. axonopodis* pv. *glycines*. Compared with its counterparts in the vascular pathogens *X. oryzae* pv. *oryzae* and *X. campestris* pv. *campestris* (de Crecy-Lagard et al. 1990; Guan et al. 2009; He et al. 2007), the Clp homolog of the parenchyma pathogen *X. axonopodis* pv. *glycines* displays broader functions than those found in the vascular pathogens, possibly through the distinct interactions with its host soybean.

Consistent with the phenotypes on the plates, we found that Clp positively regulates carbohydrates utilization of *X. axonopodis* pv. *glycines* (Fig. 1). Interestingly, the multicopy (~15 copies) complementation strain (in the *clp* mutant background) displayed decreased growth compared with the wild-type strain, implying that overexpression of *clp* probably attenuates the growth of *X. axonopodis* pv. *glycines* (data not shown). To confirm this, we

overexpressed the *clp* in the wild-type background, and the *clp* overexpressing strain *X. axonopodis* pv. *glycines* (*clp*) shared similar growth rates both qualitatively and quantitatively as the multicopy complementation strain (Fig. 1), suggesting that the optimal Clp is essential for carbohydrates utilization in *X. axonopodis* pv. *glycines*. Similar to what has been observed for the *pgi* in *X. campestris* pv. *vesicatoria*, in which its expression is inhibited by its substrates (Guo et al. 2017), the expression of *clp* was significantly repressed by the carbohydrates, the utilization of which was either positively or negatively regulated by *clp*. It is possible that the carbohydrates indirectly repress the expression of *clp* through the *hrp* system in the soybean pathogen *X. axonopodis* pv. *glycines* (Guo et al. 2011, 2012a). To our knowledge, it has not been previously shown that a Clp homolog participates in the regulation of carbohydrates utilization in *Xanthomonas* spp.

Similar to the *clp* mutant of the vascular pathogens *X. campestris* pv. *campestris* and *X. oryzae* pv. *oryzae* (Guan et al. 2009; He et al. 2007), deletion of *clp* in the parenchyma pathogen *X. axonopodis* pv. *glycines* resulted in significantly reduced EPS production (Fig. 2A). However, contrasting with that in *X. campestris* pv. *campestris* and *X. oryzae* pv. *oryzae*, the EPS production was significantly reduced in the *clp*-overexpressing strain compared with the wild-type strain (Fig. 2A). The compromised expression of *gum* cluster (Fig. 2B), which is responsible for the EPS biosynthesis in *Xanthomonas* spp. (Vojnov et al. 2001; Yang et al. 2016), might account for the reduced EPS production in the *clp* mutant as well as in the *clp*-overexpressing strain. The *gum* cluster (*gumB-M*) belongs to the transcribed polycistronic messenger RNAs or the same transcriptional unit, the promoter of which is located at the upstream *gumB* coding region. However, the opposite expression of *gumB-M* genes was observed, which could be caused by the presence of small promoters in the coding region (Lee et al. 2008; Yu et al. 2015). Additionally, the reduced carbohydrates utilization by the *clp* mutant and *clp*-overexpressing strain probably could repress the pathogen to break down carbohydrates for energy and carbon molecules, both of which are also necessary for EPS production (Guo et al. 2017; Lu et al. 2009b).

Because of the unique regulatory characteristics of *X. axonopodis* pv. *glycines* Clp in the biosynthesis of EPS, which play an important

Fig. 7. The expression of Crp-like protein (*clp*) is positively regulated by **A**, the diffusible signal factor-mediated quorum sensing system and **B**, the HrpG/HrpX cascade. Integration host factor A was used as an internal reference gene for normalization. Data are means $\pm$ standard deviations from three replicates. **Significant differences at $P = 0.01$ by the Student's *t* test. Xag, *X. axonopodis* pv. *glycines*.

role in the formation of aggregates or biofilm (Dow et al. 2003), we were prompted to explore the roles for *X. axonopodis* pv. *glycines* Clp in regulating biofilm formation/dispersal. We found that, similar to EPS production, the *clp* mutant and the *clp*-overexpressing strains both exhibit a decreased biofilm formation compared with the wild-type strain (Fig. 3). Interestingly, our results are inconsistent with the previous reports that Clp homologs from the vascular pathogen *X. campestris* pv. *campestris* and *X. oryzae* pv. *oryzae* are not involved in the regulation of DSF-dependent biofilm dispersal (Guan et al. 2009; He et al. 2007). The processes that regulate aggregate formation/dispersal by QS, such as DSF and *N*-AHLs, in plant pathogenic bacteria have not been defined yet, and the molecular mechanisms are probably different in each case (Davies et al. 1998; Dow et al. 2003; Zhang and Pierson 2001). It has been reported that both the *gum* and *xagABCD* operons contribute to biofilm formation under different conditions, whereas the extracellular enzyme ManA disperse the aggregates in *Xanthomonas* spp. (Zhang et al. 2013; Zhou et al. 2013). Comparative genomic analysis reveals that only the *gum* operon and *manA* are present (but the *xagABCD* operon is not present) in the genome of *X. axonopodis* pv. *glycines*. The compromised expression of *gum* cluster (Fig. 2B) and *manA* (Fig. 6B) might

account for the reduced biofilm formation in the *clp* mutant as well as in the *clp*-overexpressing strain.

The contribution of flagellar motility to the pathogenesis of bacterial pathogens of plants varies in different species. Flagellar motility seemed to be required for biofilm formation and pathogenicity of *X. axonopodis* pv. *citri*, the causative agent of citrus canker (Malamud et al. 2011). However, flagellar motility might not be essential for the virulence of the vascular pathogen *X. oryzae* pv. *oryzae* (Tian et al. 2015). The probable reason for this is that bacterial cells are nonmotile after they are inside of the host xylem (Tans-Kersten et al. 2004). We found that deletion of *clp* in *X. axonopodis* pv. *glycines* resulted in significantly reduced cell motility, whereas overexpressing *clp* in the wild-type strain significantly increased the cell motility (Fig. 4A and B). The decreased or increased motility was highly correlated with the expressions of *fliA*, *fleN*, *flhF*, *rpoN2*, and *fleQ* (Fig. 4C), which are required for swimming motility (Tian et al. 2015). The reduced cell motility was also observed in the *clp* mutant of *X. oryzae* pv. *oryzae* strain PXO99^A (Guan et al. 2009), indicating a conserved function for *clp* in cell mobility between these two species. We also presented additional evidence that the level of intracellular ATP, which provides the energy for cell motility (Lu et al. 2009a), was

Fig. 8. Crp-like protein (*clp*) is essential for the growth and virulence of *Xanthomonas axonopodis* pv. *glycines* in host soybean. **A**, Bacterial pustule (BP) symptoms caused by *X. axonopodis* pv. *glycines* strains on the inoculated leaves of soybean seedlings (cultivar Suinong10; susceptible to BP, 6 weeks old). Photographs were taken on days 7 to 10 postinoculation. **B**, Growth of bacteria in inoculated leaves of soybean. Bacteria were recovered from the inoculated leaves at different days postinoculation. Data are means $\pm$ standard deviations from three replicates. *Xag*, *X. axonopodis* pv. *glycines*.

significantly reduced in the *clp* mutant compared with in the wild-type strain (Fig. 4D). The reduced ATP level may be one of the major findings that negatively affects cell motility in the *clp* mutant. However, whether flagellar motility contributes to the pathogenicity of *X. axonopodis* pv. *glycines* remains to be investigated in the future.

Clp positively regulates the biosynthesis of CWDEs (Fig. 6A) and negatively regulates protease activity of *X. axonopodis* pv. *glycines* (Fig. 5A). These positive and negative roles of Clp are corroborated perfectly by the correlations between levels of *clp* and the expression levels of corresponding pathway genes in different strains (Fig. 5C; Fig. 6B and C). It has been reported that the regulation of Clp in the biosynthesis of extracellular enzymes differs significantly among different *Xanthomonas* spp. For example, deletion of *clp* in *X. campestris* pv. *campestris* strain XC1 resulted in decreased cellulase activity and protease activity (He et al. 2007), whereas the *clp* mutant in *X. oryzae* pv. *oryzae* strain PXO99^A did not affect the cellulase activity (Guan et al. 2009), and deletion of *clp* in *X. campestris* pv. *campestris* strain NRRLAB1459 led to increased protease activity (de Crecy-Lagard et al. 1990). In addition, the presence of some extracellular enzymes or activities seems to be significantly distinct in different xanthomonads. For example, EngXCA is a major cellulase in *X. campestris* pv. *campestris* (Schröter et al. 2001) but not in *X. citri* subsp. *citri* 29-1 strain (Xia et al. 2016), and the protease activity exists in *X. oryzae* pv. *oryzae* strain T7174R (Lu et al. 2011) but not in *X. oryzae* pv. *oryzae* strain PXO99^A (Zhou et al. 2013).

As expected in *X. campestris* pv. *campestris* and *X. axonopodis* pv. *citri* (Guo et al. 2011; He et al. 2007), the expression of *clp* in *X. axonopodis* pv. *glycines* is positively regulated by the DSF-mediated QS system and the HrpG/HrpX cascade in the rich NYG medium (Fig. 7A and B). In addition, the expression of *clp* is also positively regulated by the HrpG/HrpX cascade in apoplast-mimicking XVM2 medium and soybean sap (Supplementary Fig. S8). All of these results suggested a possibility that similar upstream regulatory mechanisms may exist among the three strains for *clp* expression. In the *X. campestris* pv. *campestris* ATCC33913 strain, the promoter region of *clp* contains a putative DNA binding site GATGA-N6-CCACA (cutoff score = 0.7) (He et al. 2007), which could be specially recognized by Clp, indicating that *clp* might be regulated directly by itself. However, sequence analysis revealed that the promoter region of *clp* in *X. axonopodis* pv. *glycines* does not contain such a DNA binding site. Furthermore, the GUS activity assay also revealed that the expression of *clp* was not self-regulated in *X. axonopodis* pv. *glycines* (Supplementary Fig. S9), indicating that the regulation of *clp* expression between *X. axonopodis* pv. *glycines* and *X. campestris* pv. *campestris* could be different. It is worth mentioning that the optimal level of Clp is necessary for maintaining both the virulence and the growth in planta for *X. axonopodis* pv. *glycines* (Fig. 8). The attenuation in virulence and growth of either the knockout or the *clp* overexpression strain in soybean leaves may have resulted, at least partially if not completely, from the combined effects of the compromised carbohydrates utilization (Fig. 1) and EPS production (Fig. 2A), reduced protease activities (Fig. 5A), and the Clp-dependent cell motility (Fig. 4A) as well as the impaired CWDEs synthesis (Fig. 6A). Nutrient utilization is essential for the pathogen to grow and colonize within the host cells (Mellgren et al. 2009; Tang et al. 2005). Therefore, the reduced ability of either the *clp* knockout or overexpression strain to utilize available carbohydrates in the host reduces its growth and aggressiveness. Consistent with this, we have previously shown that the *zwf* (encoding glucose-6-phosphate dehydrogenase) and *pgi* (encoding glucose 6-phosphate isomerase) mutants in *X. campestris* pv. *vesicatoria*, both of which lose the capability to utilize hexose or pyruvate as the sole carbon sources, have limited growth in host tissues and significantly reduced the virulence (Guo et al. 2015, 2017). EPS has been shown to act as an important virulence factor that plays a prominent role during

pathogen infection in *Xanthomonas* spp. (Guo et al. 2015; Yang et al. 2007). It can enhance the susceptibility of the host plant by suppressing defense responses, such as callose deposition (Yun et al. 2006), mask the bacterium to prevent recognition by the host (Alvarez 2000), and contribute to biofilm formation (Dow et al. 2003). It has been shown that inactivation of the glyceraldehyde-3-phosphate dehydrogenase, which can result in reduction of EPS production, significantly reduced the virulence of *X. campestris* pv. *campestris* (Lu et al. 2009b). It was also reported that cell motility makes a contribution to the invasion and colonization of the pathogen and the development of disease symptom (Guo et al. 2015; Lu et al. 2009a). Although the relationship between cell motility and virulence has not been specifically reported, cell motility and virulence factor EPS could have crosstalk (Lu et al. 2009a). Thus, we speculate that cell motility could contribute to the full virulence of *Xanthomonas* spp. to a certain degree. CWDEs secreted by the type II secretion system are essential for pathogens in virulence (Wang et al. 2008; Zou et al. 2012). During pathogenesis, plant cell walls act as the first barrier of defense against bacterial invasion and multiplication. The enzymatic activities of CWDEs may facilitate pathogen invasion into the cells of its host plants by digesting plant cell structures, thus contributing to disease development (Xia et al. 2016; Zou et al. 2012). The intensity of extracellular protease activity in bacterial leaf streak pathogenic strains has been used as an indicator for virulence assessment in rice (Zou et al. 2012). Consistent with this, it has been shown that EcpA, an extracellular protease, is a specific virulence factor required by the parenchyma pathogen *X. campestris* pv. *vesicatoria* (Zou et al. 2012).

Our data together with the results from other studies enable us to propose a working model to depict how Clp coordinates the DSF-mediated signals and the HrpG/HrpX signaling cascade that ultimately regulate multiple biological properties. In this model (Supplementary Fig. S10), DSF signals generated by RpfF are transduced through the RpfC sensor to the RpfG response regulator via a conserved phosphorelay mechanism (Cai et al. 2017; Ryan et al. 2006; Slater et al. 2000). Through controlling the intracellular level of c-di-GMP, the congenital ligand of the global transcription factor Clp, RpfG modulates the regulatory activity of Clp (Leduc and Roberts 2009; Tao et al. 2010). Parallel to RpfC/RpfG two-component regulatory system, the RpfS/RpfR system also could sense and transduce DSF signals (An et al. 2014). Additionally, the RavS/RavR system senses and transduces low-oxygen signals (He et al. 2009), and HrpX/HrpG system transduces environmental signals recognized by an unidentified receptor or sensor (Zhang et al. 2009). All of these signals converge on and positively regulate the *clp* expression or activity. As a consequence, the increased level of *clp* and/or activated Clp results in altered expressions of multiple clusters of downstream genes and ultimately, modulates numerous biological processes, such as carbohydrates utilization, EPS production, biofilm formation, cell motility, and synthesis of extracellular enzymes, which are essential for the full virulence and adaptation of the parenchyma pathogen *X. axonopodis* pv. *glycines* in soybean. The convergence and integration of various signals to the global regulator Clp enable *X. axonopodis* pv. *glycines* to coordinate these signals and respond to the external changes with high efficiency and great flexibility. In sum, our study has significantly advanced the understanding of the roles for the global regulator Clp of *Xanthomonas* spp.

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