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ORIGINAL ARTICLE

The phytopathogen *Dickeya dadantii* 3937 *cpxR* locus gene participates in the regulation of virulence and the global c-di-GMP network

Daqing Jiang[1] | Quan Zeng[2] | Biswarup Banerjee[1] | Haiping Lin[3] | John Srok[1] | Manda Yu[1] | Ching-Hong Yang[1]

[1]Department of Biological Sciences, University of Wisconsin-Milwaukee, Milwaukee, Wisconsin, USA

[2]Department of Plant Pathology and Ecology, The Connecticut Agricultural Experiment Station, New Haven, Connecticut, USA

[3]School of Forestry and Biotechnology, Zhejiang Agricultural and Forestry University, Hangzhou, China

Correspondence
Manda Yu, Department of Biological Sciences, University of Wisconsin-Milwaukee, Milwaukee, WI, USA.
Email: yu32@uwm.edu

Ching-Hong Yang, Department of Biological Sciences, University of Wisconsin-Milwaukee, Milwaukee, WI, USA.
Email: chyang@uwm.edu

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Abstract

Bacteria use signal transduction systems to sense and respond to their external environment. The two-component system CpxA/CpxR senses misfolded envelope protein stress and responds by up-regulating envelope protein factors and down-regulating virulence factors in several animal pathogens. *Dickeya dadantii* is a phytopathogen equipped with a type III secretion system (T3SS) for manipulating the host immune response. We found that deletion of *cpxR* enhanced the expression of the T3SS marker gene *hrpA* in a designated T3SS-inducing minimal medium (MM). In the Δ*cpxR* mutant, multiple T3SS and c-di-GMP regulators were also up-regulated. Subsequent analysis revealed that deletion of the phosphodiesterase gene *egcpB* in Δ*cpxR* abolished the enhanced T3SS expression. This suggested that CpxR suppresses EGcpB levels, causing low T3SS expression in MM. Furthermore, we found that the Δ*cpxR* mutant displayed low c-di-GMP phenotypes in biofilm formation and swimming. Increased production of cellular c-di-GMP by in trans expression of the diguanylate cyclase gene *gcpA* was negated in the Δ*cpxR* mutant. Here, we propose that CpxA/CpxR regulates T3SS expression by manipulating the c-di-GMP network, in turn modifying the multiple physiological activities involved in the response to environmental stresses in *D. dadantii*.

KEYWORDS
c-di-GMP, CpxR, *Dickeya dadantii*, phytopathogens, T3SS

1 | INTRODUCTION

The phytopathogen *Dickeya dadantii* 3937 (previously named *Erwinia chrysanthemi* 3937), a gram-negative bacterium, was initially isolated from a lesion spot of a wilted African violet (*Saintpaulia ionantha*) and characterized in 1953 (Burkholder et al., 1953). Later research revealed *D. dadantii* has a wide range of hosts and is capable of infecting many economically important plants, including potatoes, tomatoes, and cabbages (Czajkowski et al., 2011). The typical symptom caused by *D. dadantii* is soft rot, which occurs at the site of infection. The macerated plant tissue releases a substantial amount of oligosaccharides, providing nutrients for the bacteria to multiply.

The bacteria also have an epiphytic lifestyle when they reside on the surface of leaves or underground in water. Because of their ability to adapt to various nutrient conditions, they have become an emerging threat to crop production, and prevention strategies are required to address *D. dadantii* infection.

Bacterial pathogens rely primarily on signal transduction systems to adapt to different environments and tackle host immune responses. One-component systems along with two-component systems (TCSs) are known to sense and respond to a myriad of environmental signals and correspondingly change the bacterial transcriptional profile. A basic stimulus-response from a classical TCS involves a membrane-associated sensor kinase (SK) that catalyses three reactions: autophosphorylation, phosphorylation, and dephosphorylation (Zschiedrich et al., 2016). Depending on different TCSs, activated SK is able to phosphorylate or dephosphorylate its corresponding response regulator receiver domain (REC) (Mizuno, 1998). Response regulators can mediate the cellular response, mostly by altering the bacterial transcriptional profile (Zschiedrich et al., 2016). On average, the bacterial genome encodes 30 TCSs (Schaller et al., 2011). There are exceptions; for example, *Myxococcus xanthus* has over 200 TCSs (Shi et al., 2008). An examination of the *D. dadantii* genome revealed the presence of 30 TCSs (Yap et al., 2008).

The sensing mechanisms also contribute to pathogenesis through motility and chemotaxis (Prüß, 2017), driving the cell into plant tissues through natural openings or wounds and further towards the apoplast, where they can digest polysaccharides from plant cell walls through a battery of plant cell wall-degrading enzymes. The type III secretion system (T3SS), an envelope-spanning complex, is regulated by at least two TCSs, HrpX/HrpY and GacA/GacS, in *D. dadantii*. The TCS HrpX/HrpY transcriptionally activates T3SS expression by increasing the transcription of *hrpL*, encoding the T3SS master regulator HrpL (Wei & Beer, 1995). The transcription initiation of *hrpL* requires the binding of RpoN (a sigma factor σ^{54}) to an RNA polymerase and an RpoN enhancer-binding protein HrpS. The transcription of HrpS is activated by the TCS HrpX/HrpY (Tang et al., 2006). The *hrpL* transcripts are also subjected to posttranscriptional regulation by RsmA, which binds to *hrpL* mRNA and promotes its degradation. On the other hand, the TCS GacA/GacS activates the transcription of RsmB regulatory RNA that binds to the RsmA protein and neutralizes the negative effect of RsmA on *hrpL* mRNA. In turn, the alternative sigma factor HrpL binds to the *hrp* box region (GGAACC-$N_{15/16}$-CCACNNA) and activates the transcription of *hrpA* and *hrpN* (Yang et al., 2010). *hrpA* encodes the T3SS needle protein and *hrpN* encodes a harpin. The *D. dadantii* T3SS also contributes to multicellular behaviours, including pellicle formation, a cell aggregation behaviour observed within the laboratory culture surface–liquid–air interface in enterobacteria (Mee-Ngan et al., 2005).

All membrane-associated TCSs and secretion systems are located in the cell envelope. In gram-negative bacteria, the envelope consists of three parts with a periplasmic space in between two lipid bilayers: the outer membrane and the inner membrane. The cell envelope is the central hub for exchanging materials and signals between extracellular and intracellular environments. The TCS CpxA/CpxR monitors envelope perturbance and is essential to maintain envelope health. CpxA/CpxR is a classical TCS with the inner membrane-located histidine kinase CpxA and the cytoplasmic response regulator CpxR. The conserved aspartate at residue 51 of CpxR receives a phosphoryl group from CpxA and alters the transcription of a diverse range of Cpx regulon genes. The *cpxP* locus is one of the most strongly induced transcriptional loci under Cpx-inducing conditions and is frequently used as a Cpx response indicator (MacRitchie et al., 2012). In *Escherichia coli*, CpxP serves both as an inhibitor of CpxA autophosphorylation and as a P-pilus-binding protein that sends misfolded P-pilus proteins for degradation by the periplasmic protease DegP, whose transcription is also Cpx-dependent (Danese & Silhavy, 1998; Danese et al., 1995; Raivio et al., 2000).

A previous study demonstrated that the TCS CpxA/CpxR is involved in the early stages of the infection process and motility in *D. dadantii* (Bontemps-Gallo et al., 2015), yet the details of its downstream regulation have not been explained. In *D. dadantii*, the bacterial second messenger bis-(3′-5′)-cyclic dimeric guanosine monophosphate (c-di-GMP) serves as a global signalling molecule that regulates T3SS expression via multiple c-di-GMP-related components (Yi et al., 2010; Yuan et al., 2015, 2018, 2019). Cellular c-di-GMP levels are regulated by two types of enzymes: GGDEF domain-containing diguanylate cyclases (DGCs) and EAL or HD-GYP domain-containing phosphodiesterases (PDEs), which synthesize and degrade the second messenger, respectively. c-di-GMP acts as a versatile ligand that is capable of binding to PilZ domain proteins, degenerate GGDEF or EAL domain proteins, or RNA riboswitches to modulate a specific output (Hengge, 2009). Hence, the dynamic intracellular c-di-GMP concentrations enable the cell to adequately respond to its environment. Genomic analysis of the *D. dadantii* genome revealed 12 DGCs, 2 PDEs, and 2 dual-domain proteins (GGDEF and EAL domains). A collection of deletion mutants of DGCs and PDEs displayed various phenotypes, suggesting some DGCs/PDEs have specific roles in certain cellular behaviours. For example, *D. dadantii* GcpA (a DGC) controls pectate lyase (Pel) production via GcpA-mediated repression of H-NS, which is in the same pathway that up-regulates RsmB and down-regulates RsmA (Yuan et al., 2018). However, GcpA-mediated T3SS expression occurs via a different route that bypasses the H-NS and Rsm system (Yuan et al., 2018).

In this study, we unveil the link between the Cpx signalling cascade and c-di-GMP in the regulation of the T3SS. In contrast to the lysogeny broth (LB) medium used in a previously published CpxR-related T3SS study in the animal pathogen *Yersinia pseudotuberculosis* (Liu et al., 2012), the plant apoplast-mimicking minimal medium (MM) is used to induce and study T3SS expression. We demonstrate the negative effects of CpxR on T3SS expression and the involvement of CpxR in the transcription of several T3SS regulators such as HrpS and RpoN. This study proposes that the virulence, biofilm, and motility phenotypes linked to *cpxR* deletion are mediated through downstream c-di-GMP regulators. We exemplify the involvement of another TCS in the already sophisticated T3SS regulatory

mechanism, bringing us closer to elucidating how the T3SS functions under various stresses in phytopathogens.

2 | RESULTS

2.1 | CpxR is a negative regulator of T3SS expression

To study T3SS regulation, two genes encoding the needle subunit (HrpA) and a harpin protein (HrpN) of the T3SS protein complex were selected for expression analysis. Previously, enhanced *hrpA* expression was demonstrated in the diguanylate cyclase *gcpD* mutant (authors' unpublished data). We conducted a transposon screening for identification of any Δ*gcpD*-mediated T3SS regulators based on a *hrpA*::*GFP* reporter system. A total of 2057 transposon mutants were screened, and 51 potential candidates showed altered *hrpA* expression (Table S3). A transposon mutant that demonstrated a significant increase in *hrpA* expression was selected for further analysis. The insertion site was found to be within the *cpxR* gene, which encodes a response regulator from the typical TCS CpxA/CpxR. To further verify the relationship between CpxR and *hrp* gene expression, we constructed a *cpxR* deletion mutant (Δ*cpxR*), and its impact on T3SS expression was examined. A significant increase of *hrpA* and *hrpN* expression was observed in Δ*cpxR*, and the enhanced T3SS expression was restored to wild-type (WT) levels by complementation of a *cpxR* gene in a low copy number plasmid (pCL1920-*cpxR*) (Figure 1a,b). Because CpxR regulates T3SS in the WT background, we further investigated the regulatory effect of CpxR in WT *D. dadantii*.

The expression of T3SS is tightly regulated and involves multiple regulatory cascades. The T3SS master regulator HrpL is an alternative sigma factor that activates *hrp* gene transcription by recruiting RNA polymerase to a *hrp* box region (GGAACC-$N_{15/16}$-CCACNNA) (Tampakaki et al., 2010). Deletion of the *cpxR* locus resulted in an increase in *hrpL* transcription (Figure 1c). The expression of *cpxR* in trans restored *hrpL* promoter activity back to WT levels (Figure 1c), indicating that CpxR participates in T3SS repression through regulation of *hrpL* transcription in *D. dadantii*.

2.2 | Transcription of key T3SS regulators was increased in the Δ*cpxR* mutant

To further identify the potential CpxR-regulated T3SS regulators, several upstream T3SS regulators were selected, and their transcription was analysed upon deletion of *cpxR*. Studies showed that at least two regulatory cascades regulate *hrpL* at the transcriptional and posttranscriptional levels. The transcriptional regulation of *hrpL* is through HrpS, which facilitates the binding of RpoN to the promoter region of *hrpL* (Tang et al., 2006). We examined *hrpS* and *rpoN* promoter activity in the Δ*cpxR* mutant. At 24 h, transcription of both *hrpS* and *rpoN* was significantly increased in the Δ*cpxR* mutant (Figure 2a,b). This observation indicates that CpxR controls T3SS expression through regulating transcription of *hrpS* and *rpoN*.

The TCS GacA/GacS regulates T3SS through elevating the transcription of the regulatory small RNA (sRNA) RsmB. RsmB binds to RsmA and neutralizes the activity of RsmA on *hrpL* mRNA degradation (Chatterjee et al., 2002; Liu et al., 1998). The promoter activities of *rsmA* and *rsmB* were therefore examined. Although RsmA and RsmB are expected to work counteractively in T3SS expression, both *rsmA* and *rsmB* transcription was increased in Δ*cpxR* (Figure 2c,d). This posed a puzzle in identifying the role of CpxR on T3SS regulation. Because the up-regulated *rsmB* might abolish the negative effect of RsmA, we tried to explain this discrepancy by examining Pel activity, which is also subject to regulation by the RsmA/RsmB system (Yuan et al., 2015). RsmA promotes the degradation of Pel mRNA, while the RsmB sRNA binds to RsmA to neutralize its negative effect on Pel mRNA (Liu et al., 1997). If RsmA/RsmB plays a major role in T3SS regulation through CpxR, higher Pel activity would be expected in the Δ*cpxR* mutant. Total Pel activity was measured spectrophotometrically to determine the net effect of the Rsm system in T3SS-inducing MM. In Δ*cpxR*, a slight but significant reduction of Pel activity was observed (Figure 2e). This result is supported by the reduced Pel activity in Δ*cpxR* through virulence assays we conducted on potato tubers. The production of Pel is essential for the phytopathogen to degrade plant cell walls (Hugouvieux-Cotte-Pattat, 2016). The reduced virulence of the Δ*cpxR* pCL1920 strain on potato tubers was recovered in complemented Δ*cpxR* pCL-*cpxR*, which showed similar maceration to the WT strain (Figure 2f). Because inverse phenotypes were observed in T3SS and Pel in Δ*cpxR*, the above results demonstrate that RsmA/RsmB does not play a major role in T3SS regulation through CpxR. This also indicates CpxR may regulate Pel through other regulator(s), not the Rsm system.

2.3 | CpxR regulates T3SS expression through CpxP in MM

Our next step was to link any Cpx response to the T3SS. Apart from MM, we also included the nutrient-rich LB medium for comparison (Bontemps-Gallo et al., 2015). Nutrient-rich media result in basal T3SS expression in phytobacteria. As reported in other studies, *cpxP* and *degP* are two Cpx regulon genes (Bontemps-Gallo et al., 2015; Price & Raivio, 2009). The promoter regions of *cpxP* and *degP* were amplified by PCR and ligated into the pPROBE-AT vector encoding green fluorescent protein (GFP). The promoter activity was measured by determining fluorescence intensity through flow cytometry similar to previous promoter activity measurements. Our Δ*cpxR* mutant showed no expression of *cpxP* in either LB or MM (Figure 3a,b). CpxP is one of the most highly induced Cpx components (Price & Raivio, 2009), and a further enhancement of *cpxP* transcription was observed in LB by in trans expression of *nlpE*, which encodes a known Cpx pathway activator (Snyder et al., 1995) (Figure 3b). However, the NlpE-induced Cpx

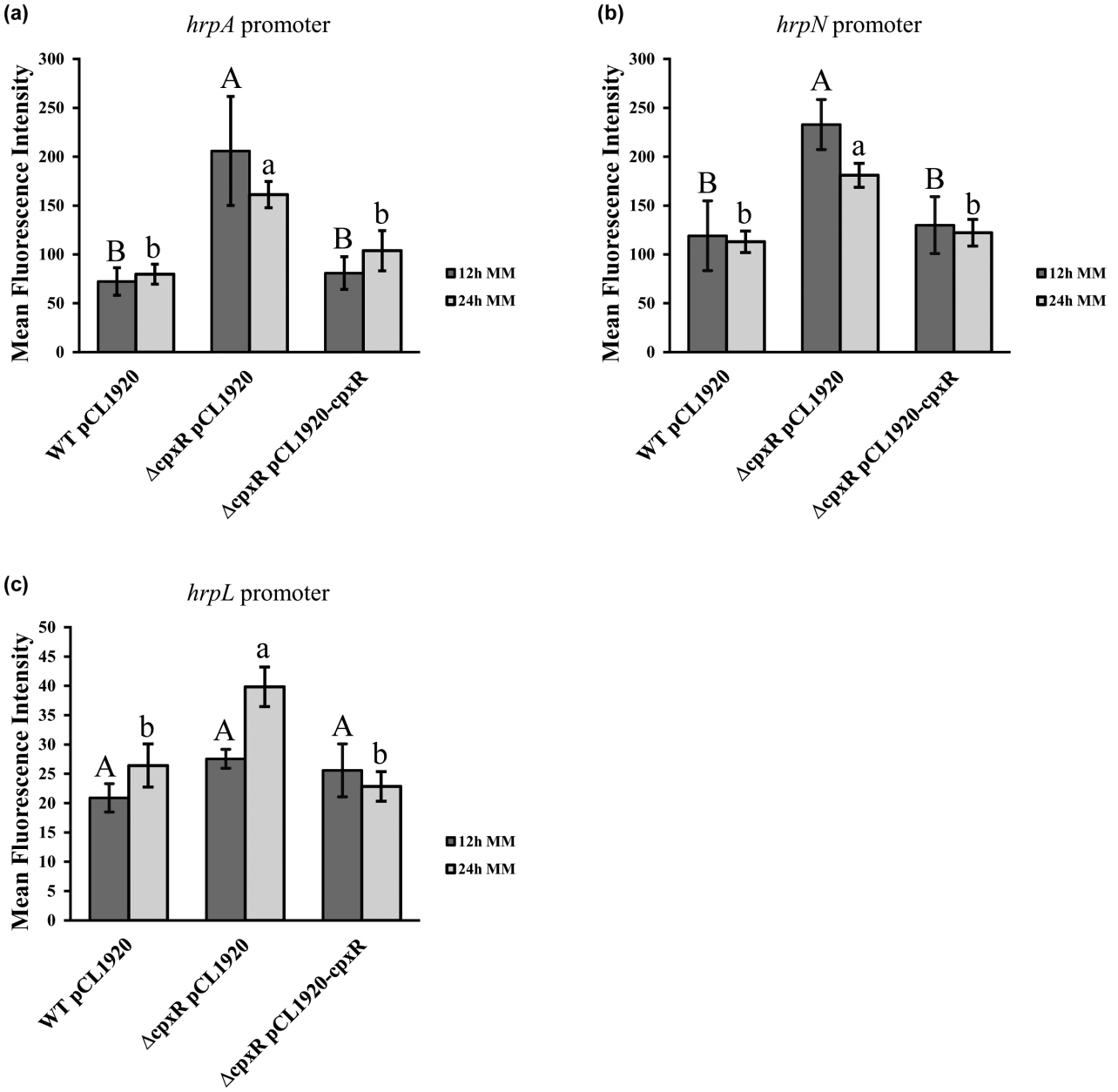

FIGURE 1 Transcriptional study of the effects of CpxR on type III secretion system (T3SS) expression in *Dickeya dadantii*. Cells grown in minimal medium (MM) for 12 and 24 h were collected and their promoter activity was measured using flow cytometry. (a,b) The transcription of two representative T3SS components, HrpA and HrpN, was examined. Wild-type (WT) strains harbouring empty vector pCL1920 were compared with Δ*cpxR*-pCL1920 and Δ*cpxR* pCL1920-*cpxR*. (c) Expression of the T3SS regulator HrpL was examined as described above. Three independent experiments were performed with three replicates for each sample ($n = 3$), and a representative figure is presented. Error bars indicate standard error of the mean. Different upper or lower case letters indicate statistically significant differences ($p < 0.05$, one-way analysis of variance)

response was insignificant in MM (Figure 3a). A complementation strain harbouring pCL1920-*cpxR* restored *cpxP* transcription to WT levels, while pCL1920-*cpxR*^{D51A}, harbouring a point mutation at the phosphorylation site of CpxR, was incapable of restoring *cpxP* transcription (Figure 3a,b). *degP* transcription was suppressed in Δ*cpxR* in LB only (Figure 3c,d) and slightly induced by NlpE in LB (Figure 3d). Deletion of *cpxR* did not fully abolish the expression of *degP*, suggesting *degP* expression is not solely dependent on CpxR. Because there was no observable change of *degP* transcription in Δ*cpxR* in the T3SS-inducing MM, the suppressive effect of CpxR on T3SS expression may not be mediated by DegP in *D. dadantii*. On the other hand, the necessity of the presence of CpxR for *cpxP* transcription suggests that the *cpxP* locus may be involved in T3SS regulation. Thus, a follow-up experiment including Δ*cpxR* with overexpressed *cpxP* was conducted. In trans expression of *cpxP* in Δ*cpxR* was able to suppress *hrpA* expression at levels similar

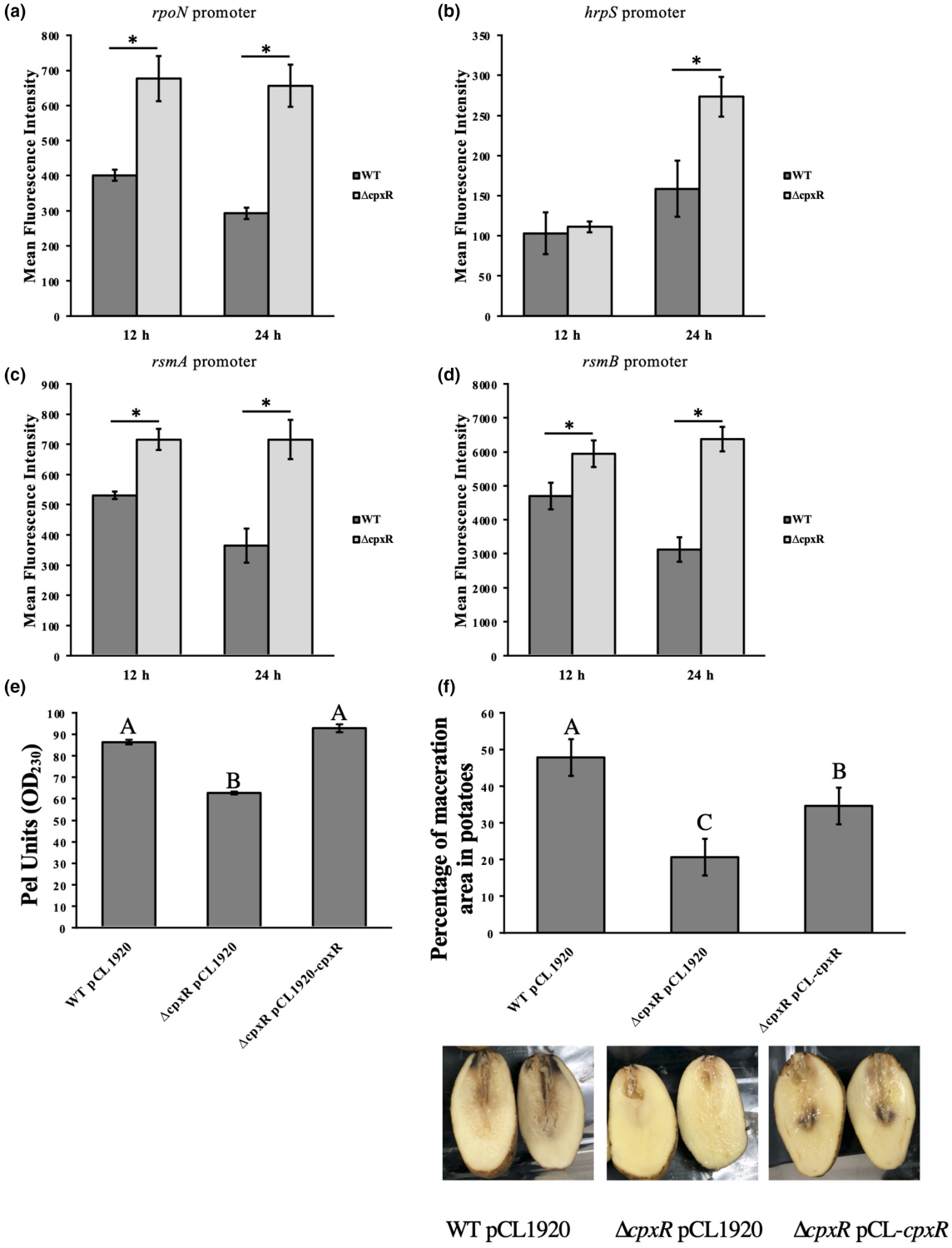

FIGURE 2 The effect of Δ*cpxR* on type III secretion system (T3SS) expression and pectinase production. (a,b) The promoter activities of *rpoN* and *hrpS* were examined in *Dickeya dadantii* and Δ*cpxR*. (c,d) The transcription of *rsmA* and *rsmB* in wild-type (WT) and *cpxR* deletion backgrounds. Strains were cultured in lysogeny broth (LB) and then transferred to minimal medium (MM) for T3SS induction. Asterisks indicate statistically significant differences ($p < 0.05$, Student's *t* test). (e) Pectate lyase (Pel) assay. WT strains were compared to Δ*cpxR* pCL1920 and complemented Δ*cpxR* pCL1920-*cpxR*. The cells were transferred from an overnight culture grown in LB to MM plus 0.1% polygalacturonic acid to induce Pel production. (f) Virulence assay performed on potato tubers with three strains. Representative maceration zones for WT, Δ*cpxR*, and complemented Δ*cpxR* are shown. Different upper case letters indicate statistically significant differences ($p < 0.05$, one-way analysis of variance). Three independent experiments were performed with three replicates for each sample ($n = 3$), and a representative figure is presented. Error bars indicate standard error of the mean

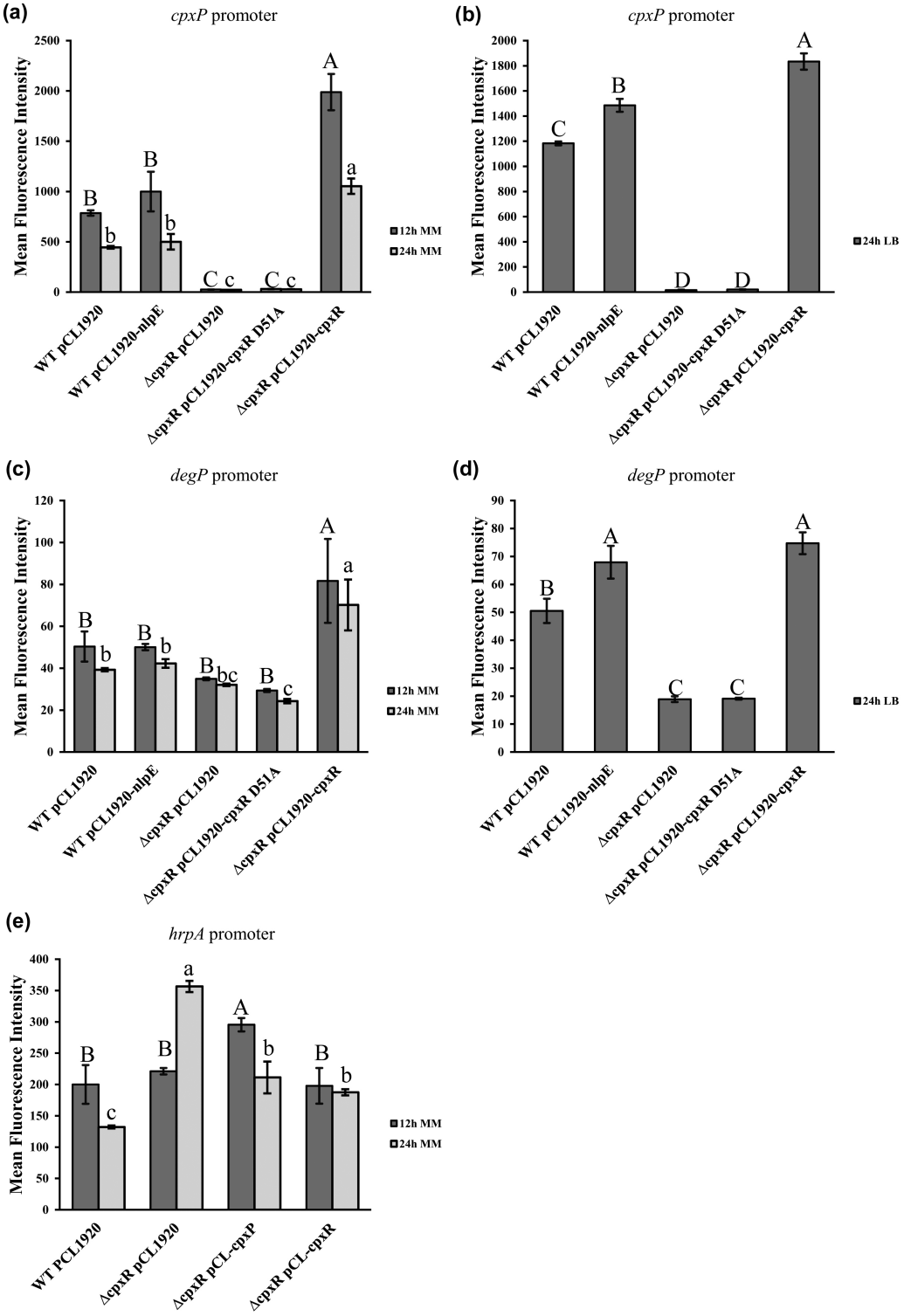

FIGURE 3 The effect of the *Dickeya* Cpx response on known CpxR targets was examined in lysogeny broth (LB) and minimal medium (MM). (a–d) Transcription of the *Dickeya dadantii cpxP* and *degP* loci was measured in wild-type (WT), Δ*cpxR* harbouring empty vector, and Δ*cpxR* complemented with pCL1920-*cpxR* or pCL1920-*cpxR*^{D51A} in LB and MM. (e) Comparison of *hrpA* expression with in trans expression of *cpxP* in Δ*cpxR*. Three independent experiments were performed with three replicates for each sample ($n = 3$), and a representative figure is presented. Error bars indicate standard error of the mean. Different upper or lower case letters indicate statistically significant differences ($p < 0.05$, one-way analysis of variance)

to in trans expression of *cpxR* in Δ*cpxR* at 24 h (Figure 3e). This further demonstrates that *cpxP* is involved in T3SS expression in *D. dadantii*.

2.4 | CpxR controls T3SS expression through manipulation of genes affecting c-di-GMP turnover

Our previous investigation of T3SS regulation showed a strong link between several T3SS regulators and bacterial second messenger c-di-GMP signalling. Several c-di-GMP components are known to participate in T3SS expression (Yi et al., 2010; Yuan et al., 2018). GcpA, a diguanylate cyclase, is able to increase intracellular c-di-GMP concentrations to down-regulate T3SS expression (Yuan et al., 2018). EGcpB and EcpC, two PDEs that catalyse hydrolysis of c-di-GMP, up-regulate T3SS expression (Yi et al., 2010). We were interested in determining if CpxR regulates T3SS expression through c-di-GMP. We first examined the effect of *cpxR* deletion on the transcription of several DGCs and PDEs. Interestingly, the results showed that *gcpA*, *egcpB*, and *ecpC* expression was increased in Δ*cpxR* (Figure 4a–c). DGCs and PDEs have contradictory effects on cellular c-di-GMP levels, which raised the question of what could be causing the net increase in T3SS expression in Δ*cpxR*. We then analysed intracellular c-di-GMP levels through a c-di-GMP reporter that harbours a transcriptional fusion of β-glucuronidase to a 110-nucleotide Vc2 RNA riboswitch, a high-affinity c-di-GMP aptamer (Sudarsan et al., 2008). The result showed that there was no significant difference in c-di-GMP levels between WT and Δ*cpx*R in MM (Figure 4d). To verify the potential strength of Δ*cpxR*-mediated T3SS expression and c-di-GMP signalling, we sought to confirm two c-di-GMP associated phenotypes in nutrient-limiting media: biofilm formation and motility. The induced motility and the repressed biofilm formation observed in Δ*cpxR* (Figure 5a,b) led us to speculate whether artificial manipulation of intracellular c-di-GMP would alter the c-di-GMP-related phenotypes in Δ*cpxR*. First, we compared *hrpA* promoter activity in *egcpB* and *ecpC* mutants. The single mutants of Δ*ecpC* and Δ*egcpB* repressed *hrpA* promoter activity (Figure 5c), which as reported before is due to the increased global c-di-GMP levels (Yi et al., 2010). The double mutants Δ*cpxR*Δ*egcpB* and Δ*cpxR*Δ*ecpC* had significantly different levels of *hrpA* promoter activity (Figure 5c). Deletion of *cpxR* in Δ*ecpC* showed increased *hrpA* activity, especially at 24 h, while no significant difference was observed between Δ*egcpB* and Δ*cpxR*Δ*egcpB* (Figure 5c). Deletion of *cpxR* did not increase *hrpA* expression in Δ*egcpB*, indicating that *hrpA* expression in Δ*cpxR* is dependent on EGcpB. To further strengthen our comprehension of the Δ*cpxR*-regulated c-di-GMP cascade, we increased the level of c-di-GMP by in trans expression of *gcpA* (pCL-*gcpA*) in WT and Δ*cpxR*. While the WT strain harbouring pCL-*gcpA* showed increased c-di-GMP levels, there was no difference in c-di-GMP levels between WT and Δ*cpxR* harbouring pCL-*gcpA* (Figure 5d). This observation

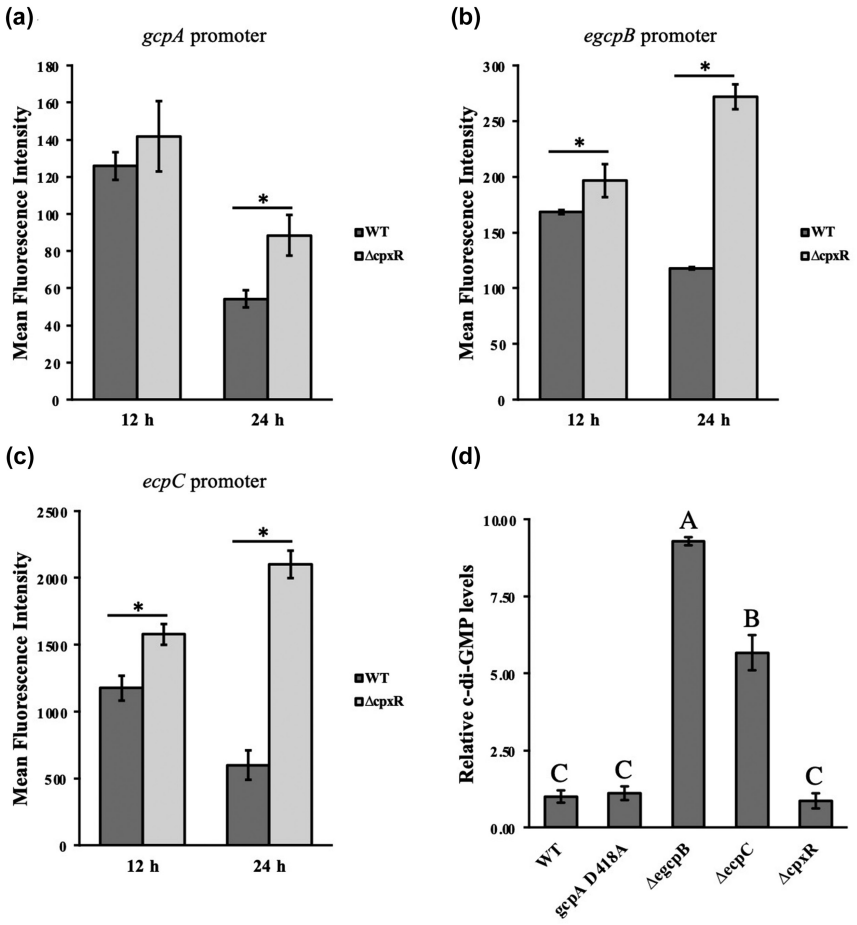

FIGURE 4 Effect of CpxR on GcpA, EGcpB, and EcpC transcription. (a–c) Effects of deletion of *cpxR* on the transcription of the diguanylate cyclase GcpA and the phosphodiesterases EGcpB and EcpC. Promoter activities were examined in minimal medium for 12 and 24 h. Asterisks indicate statistically significant differences ($p < 0.05$, Student's *t* test). (d) Relative c-di-GMP levels were examined in wild-type (WT) *Dickeya dadantii* and its derivatives *gcpA*^{D418A}, Δ*egcpB*, Δ*ecpC*, and Δ*cpxR*. The data are presented as β-glucuronidase activity values of each gene relative to the WT. Different upper case letters indicate statistically significant differences ($p < 0.05$, one-way analysis of variance). Three independent experiments were performed with three replicates for each sample ($n = 3$), and a representative figure is presented. Error bars indicate standard error of the mean

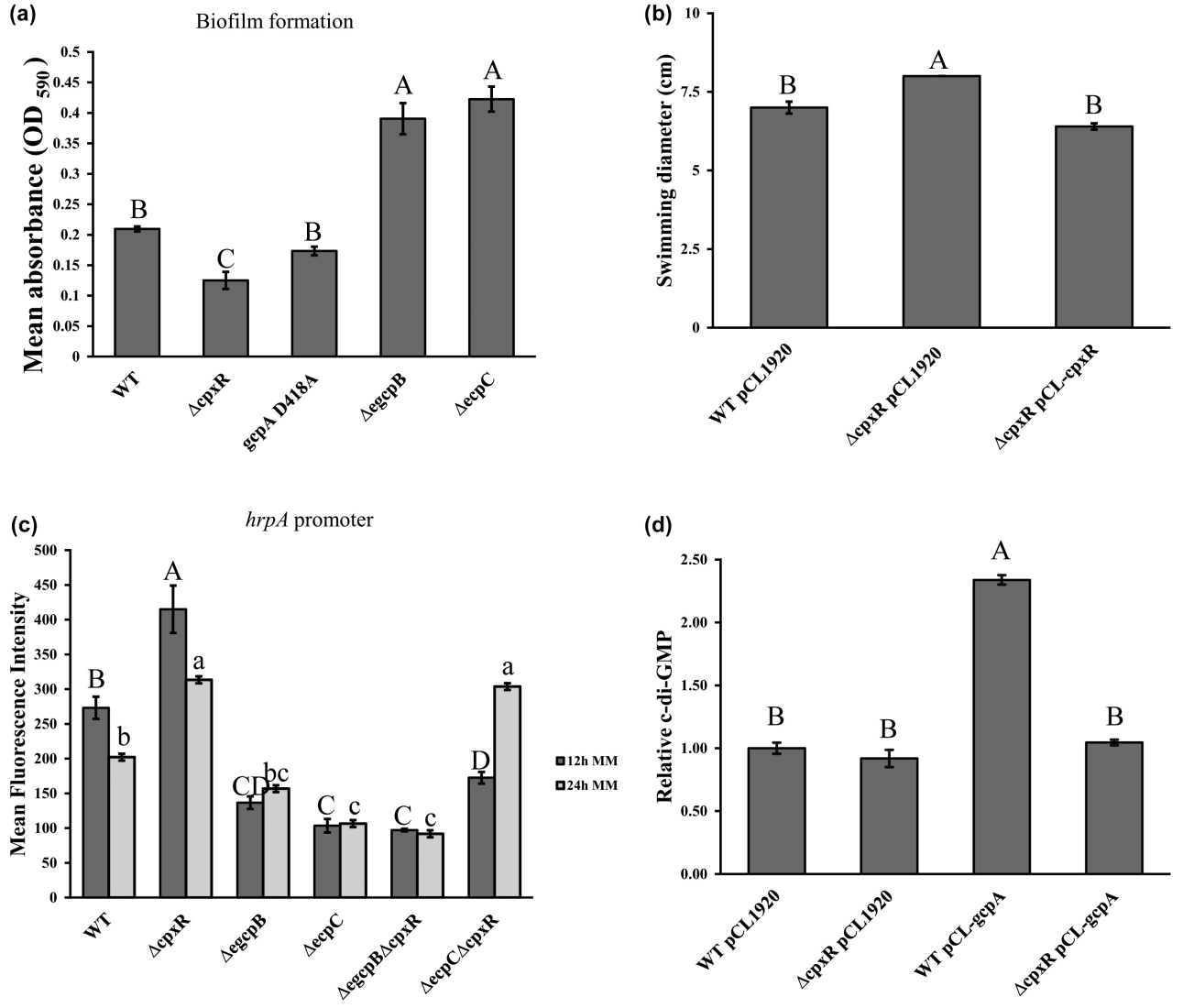

FIGURE 5 The effects of the global regulator c-di-GMP and the Cpx response on the expression of the type III secretion system (T3SS). (a) Biofilm formation of *Dickeya dadantii* wild-type (WT), Δ*cpxR*, *gcpA*^{D418A}, and phosphodiesterase mutants Δ*egcpB* and Δ*ecpC*. (b) Motility assay of *D. dadantii* WT and its derivatives Δ*cpxR* (pCL1920 or pCL1920-*cpxR*). (C) T3SS expression analysis from *hrpA* transcription analysis in WT, Δ*egcpB*, Δ*ecpC*, Δ*cpxR*, Δ*ecpC*Δ*cpxR*, and Δ*egcpB*Δ*cpxR*. (d) Relative c-di-GMP concentrations of different *D. dadantii* cells in minimal medium (MM). Values were calculated by measuring the β-glucuronidase activity from a c-di-GMP riboswitch fusion using the pRU1064-Vc2 vector. Three independent experiments were performed with three replicates for each sample ($n = 3$), and a representative figure is presented. Error bars indicate standard error of the mean. Different upper or lower case letters indicate statistically significant differences, while samples with no significant differences of the mean share common letters ($p < 0.05$, one-way analysis of variance)

suggests that the increased c-di-GMP levels by GcpA are negated by enhanced EGcpB expression in Δ*cpxR*. The Δ*cpxR* mutant harbouring empty vector showed a noticeable but insignificant reduction of c-di-GMP levels (Figures 4d and 5d). This could be because the basal level of c-di-GMP is too low to be detected by this method.

3 | DISCUSSION

Signal transduction systems provide remarkable flexibility for bacteria to respond to a variety of external and cellular signals. The CpxA/CpxR TCS is one of the most highly conserved signal transduction systems in *Enterobacteriaceae* and has been extensively studied in animal pathogens, but the regulatory role of Cpx on virulence factors in phytopathogens is rarely reported. Transposon mutagenesis and gene deletion of *cpxR* significantly increased *hrpA* and *hrpN* expression in MM, the T3SS-inducing medium of phytobacteria. Expression of two T3SS regulator genes, *rpoN* and *hrpS*, was increased in Δ*cpxR*, indicating CpxR might regulate T3SS expression via these regulators. Among the two known CpxR-regulated genes, *cpxP* and *degP* (Bontemps-Gallo et al., 2015; Price & Raivio, 2009), only *cpxP* expression was significantly reduced in the Δ*cpxR* mutant in MM, suggesting that CpxP plays a more important role in *cpxR*-mediated T3SS expression in MM. The TCS HrpX/HrpY is found in

the phytopathogen *D. dadantii* but not in animal pathogens. HrpX/HrpY positively regulates the T3SS through the HrpX/HrpY–HrpS–HrpL pathway (Tang et al., 2006). The TCS GacA/GacS is found in several *Pseudomonas* species and affects various cellular behaviours including T3SS expression (Brencic et al., 2009; Vakulskas et al., 2015). We previously reported that GacA/GacS regulates T3SS expression through the GacS/GacA–RsmB–RsmA–HrpL regulatory pathway in *D. dadantii* (Yang et al., 2008). In comparison, our results show that CpxA/CpxR regulates T3SS expression by manipulating the c-di-GMP network.

Our understanding of the Cpx response comes largely from model organisms such as *E. coli*, *Y. pseudotuberculosis*, and *Vibrio cholerae* (Acosta et al., 2015; Fei et al., 2021; Vogt et al., 2010). The initial characterization of the Cpx response showed a direct relationship to envelope stress factors such as the periplasmic proteins DegP, DsbA, and PpiA (Danese et al., 1995). Later research on the TCS CpxA/CpxR showed that the physiological role of the Cpx response was even more diverse than originally thought. For instance, a broad-scale analysis of the *E. coli* MC4100 Cpx response revealed uncharacterized targets that belong to a wide range of cellular processes (Price & Raivio, 2009). Moreover, various Cpx signal inducers were identified. These included stresses such as alkaline pH, misfolded P-pilus, and attachment to abiotic surfaces through NlpE (Danese & Silhavy, 1998; Jones et al., 1997; Snyder et al., 1995). Intracellular signals have also been shown to invoke the Cpx response, such as altered lipopolysaccharide biosynthesis (Delhaye et al., 2016) or the small phosphoryl donor acetyl-phosphate, which is generated through the AckA-Pta pathway (Wolfe, 2010) and can directly enter the Cpx pathway through CpxR phosphorylation. The broad range of Cpx targets and Cpx inducers raise the question of the specific role of the Cpx response in cellular behaviours. On top of the above findings, the T3SS-related transposon screening in this study has unveiled a novel role of CpxR in T3SS regulation in *D. dadantii*.

Although the mechanism of T3SS regulation has been reported previously, the participation of multiple layers of regulatory pathways in T3SS expression is not surprising, considering the deep complexity of molecular host–pathogen interactions. In this study, we addressed the cross-talk between T3SS expression and various signalling cascades. Our key model is based on the induced T3SS expression in plant apoplast-mimicking MM. There is a distinct difference between the study of animal pathogens and phytopathogens because most animal pathogens can express the T3SS in nutrient-rich medium. For example, the study of *Y. pseudotuberculosis* requires brain heart infusion broth, a nutrient-rich medium, to induce T3SS expression (Liu et al., 2012). The majority of Cpx studies in *Enterobacteriaceae* used LB medium, which, in contrast, suppresses T3SS expression in plant pathogens (Yang et al., 2008). In this study, the NlpE-induced Cpx response was not significant in MM when compared to LB (Figure 3a,c), indicating Cpx-mediated T3SS regulation in *D. dadantii* in MM could be different from that in the reported organisms.

Several studies indicate that CpxR participates in the regulation of secretion systems in several animal pathogens. CpxR might directly suppress the *Y. pseudotuberculosis* Ysc-Yop T3SS through binding to the promoter regions of *lcrF* and *yop* (Liu et al., 2012), where *lcrF* encodes an AraC-like transcriptional activator responsible for Ysc-Yop T3SS transcription (Cornelis et al., 1989). The direct role of CpxR binding to the Dot/Icm translocation system proteins (a type IV_B secretion system) was also observed in the study of *Legionella pneumophila* through gel mobility shift assays (Altman & Segal, 2008). Conversely, the study of enteropathogenic *E. coli* Δ*cpxR* showed a minimal effect on T3SS expression, but the CpxR-regulated protease DegP plays an essential role in enteropathogenic *E. coli* T3SS assembly at the posttranscriptional level (MacRitchie et al., 2012). The variability of Cpx responses observed in various pathogens under diverse conditions is worth noting and awaits further clarification.

We hypothesized that CpxR of *D. dadantii* might affect some global responses that alter multiple T3SS regulators such as RpoN, HrpS, or HrpL, as reported in this study. The bacterial second messenger c-di-GMP is a global regulator that regulates multiple phenotypes in *D. dadantii* (Yuan et al., 2018). The study of this single ribonucleotide also raises the question of its specificity. Global and local c-di-GMP pools have been proposed to explain the seemingly contradictory cellular behaviours (Hengge, 2021). Although the specific c-di-GMP effector(s) of the T3SS remain unknown in *D. dadantii*, surface plasmon resonance assays suggested the T3SS ATPase HrcN is a potential c-di-GMP binding target (Trampari et al., 2015). There is a possibility that CpxR binds to the promoter regions of the DGC GcpA or PDEs EGcpB and EcpC, but this hypothesis needs further confirmation. Through c-di-GMP binding riboswitch experiments, we were able to measure the relative c-di-GMP levels in MM. No significant differences in global c-di-GMP levels were observed between the WT and Δ*cpxR* (Figure 5d), so we speculate that deletion of *cpxR* might affect the T3SS through altering local c-di-GMP pools. Interestingly, in trans expression of *gcpA* in Δ*cpxR* did not result in increased c-di-GMP levels compared to the WT. There are two possibilities to explain this phenomenon: (a) the increased c-di-GMP might be accordingly degraded by the increased expression of PDEs; or (b) the function of GcpA is suppressed. Because EGcpB is a strong c-di-GMP remover (Figure 4d) and the variation of *gcpA* expression was minor (Figure 4a) in MM, we prefer the hypothesis that c-di-GMP was cleared by EGcpB in Δ*cpxR*. Although the difference of c-di-GMP levels between WT and Δ*cpxR* may be too subtle to be detected, the low c-di-GMP phenotypes such as induced swimming and repressed biofilm formation were found in Δ*cpxR* (Figure 5a,b). Pel activity is reduced in Δ*cpxR*, which is opposite to the increased T3SS expression. In many gram-negative phytopathogens, such as *Pseudomonas syringae*, T3SS is the major virulence factor for pathogenicity. *P. syringae* uses the T3SS to deliver type III effector proteins into the host to optimize pathogen growth while subverting host defence responses (Cunnac et al., 2009). In comparison, the T3SS of *D. dadantii* plays a minor role in virulence. The pectolytic *D. dadantii* uses pectinases to break down the plant cell wall and causes soft rot symptoms as the primary characteristic of virulence.

In this study, the negative effect of CpxR on *D. dadantii* T3SS expression in MM is identified, and a model for the cross-talk between

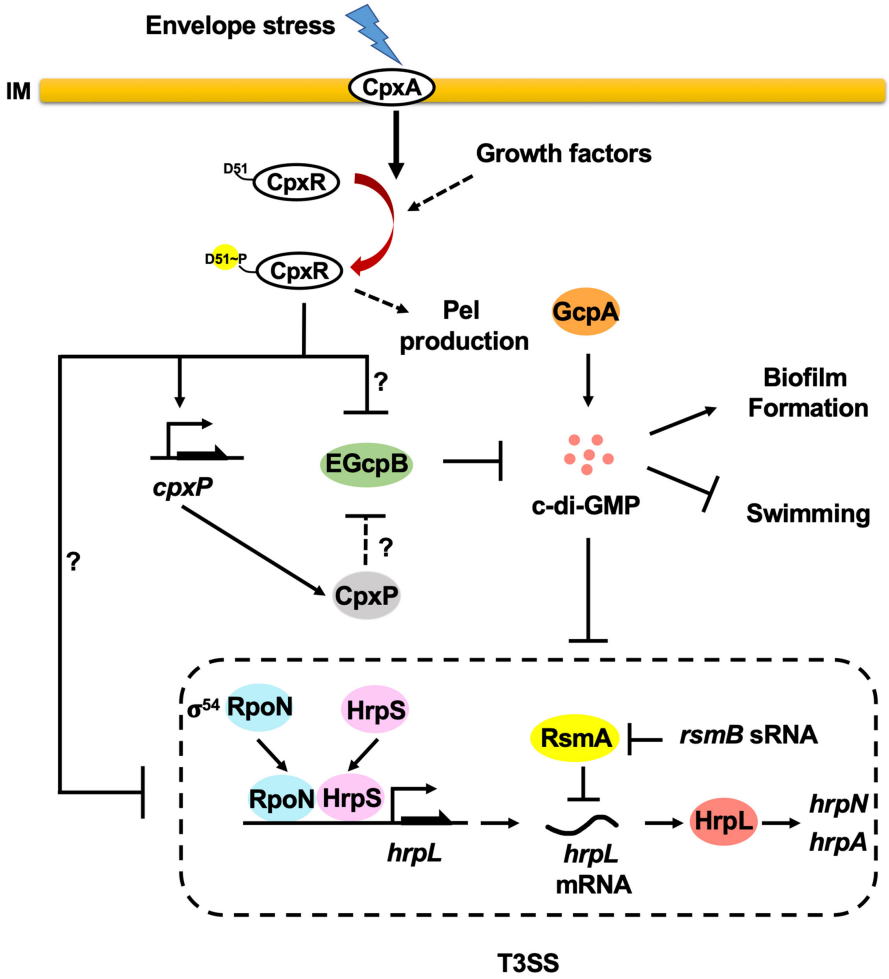

FIGURE 6 Proposed mechanism of how CpxR regulates type III secretion system (T3SS) expression in *Dickeya dadantii*. Envelope stress or growth factors activate CpxR by phosphorylation at aspartate residue D51. CpxR shows a negative effect on various T3SS regulators at the transcriptional level. CpxR positively regulates the production of pectate lyase (Pel). Activated CpxR also suppresses EGcpB, a phosphodiesterase (a c-di-GMP-degrading enzyme), through CpxP or an unknown mechanism. High c-di-GMP concentrations inhibit T3SS expression and swimming and promote biofilm formation. Deletion of *cpxR* abolishes the suppression of EGcpB and hence increases T3SS expression

CpxR and c-di-GMP in T3SS regulation is proposed (Figure 6). CpxR suppresses T3SS expression, which might be through the suppression of PDEs such as EGcpB. The diverse roles of CpxR in complex bacterial signal transduction systems suggest CpxR may serve as a potential drug target that could be used to facilitate bacterial management and disease control in the future.

4 | EXPERIMENTAL PROCEDURES

4.1 | Bacterial strains, plasmids, primers, and cultures

The bacterial strains and media used in this work are listed in Table S1. Primers are listed in Table S2. *E. coli* strains were grown in LB medium (1% tryptone, 1% NaCl, and 0.5% yeast extract) at 37°C. *D. dadantii* strains were cultured in LB medium, mannitol–glutamic acid (MG) medium (1% mannitol, 0.2% glutamic acid, 0.05% potassium phosphate monobasic, 0.02% NaCl, and 0.02% $MgSO_4$), or low nutrient T3SS-inducing MM at 28°C (Yang et al., 2008). Antibiotics were added to the culture at the listed concentrations: ampicillin (100 μg/ml), kanamycin (Km; 50 μg/ml), and spectinomycin (100 μg/ml). Genomic data of *Dickeya* were retrieved from a systematic annotation package for community analysis of genomes (ASAP) (https://asap.ahabs.wisc.edu/asap/home.php).

4.2 | Mutant construction and complementation

Marker exchange mutagenesis was used to construct Δ*cpxR* and other mutants (Yang et al., 2002). In brief, the downstream and upstream sequences of the target gene locus (e.g., the *cpxR* locus) were each amplified by PCR using specific primers (Table S2). The achieved DNA fragments were ligated with the Km cassette

fragment (c.1.5 kb) from the pKD4 plasmid (Datsenko & Wanner, 2000) using three-way cross-over PCR. The DNA fragments of the correct size were then digested and ligated into the pWM91 suicide plasmid. After conjugation using *E. coli* S17-1 λ-pir, plasmid pWM91 was transformed into *D. dadantii*. Following a selection of recombinants grown in 10% sucrose MG agar, colonies showing sucrose resistance because of the loss of SacB-mediated toxicity were then plated onto an LB ampicillin plate, and the ampicillin-sensitive cells were picked and stored at −80°C for future analysis. Successful mutant generation was confirmed by sequence analysis of the target gene using flanking primers.

To construct double mutants, removal of the Km cassette from marker exchange mutants was first performed. To remove the Km cassette, the pFLP2 plasmid encoding the FLP (flippase) recombinase enzyme in *E. coli* S17-1 λ-pir was conjugated with mutant harbouring Km cassette in LB agar. After excision of the Km cassette through the pFLP2 plasmid, Km-sensitive and sucrose-resistant strains were selected on MG agar plates and analysed by sequencing using flanking primers. The double mutant was then constructed using marker exchange mutagenesis as mentioned above. To generate complemented strains, the low copy number plasmid pCL1920 was used to express the open reading frame regions of target genes. The target region was cloned into pCL1920 and downstream of a leaky *lac* operon. The constructed vector was then transformed into *Dickeya* strains. All constructed plasmids were confirmed by sequencing and phenotype analysis.

4.3 | Transcriptional analysis using the GFP reporter plasmid

To construct the GFP reporter plasmids pAT-*cpxP*, pAT-*cpxR*, and pAT-*degP*, the promoter regions and a small portion of the open reading frames of target genes were amplified by PCR and ligated into the probe vector pPROBE-AT (Leveau & Lindow, 2001). The mean fluorescence intensity was measured by FACSCalibur flow cytometry (BD Biosciences) after culture in LB medium or MM, and bacterial cell suspensions were collected at different time points. Samples were diluted 100× using phosphate-buffered saline (PBS) and detected by flow cytometry (Peng et al., 2006). For each test, around 10,000 cells were accessed and the GFP levels of the cells were detected at an excitation wavelength of 488 nm and an emission filter of 530 nm. To study T3SS expression, the T3SS subunits HrpA and HrpN, and the T3SS master regulator HrpL were chosen. pAT-*hrpA*, pAT-*hrpN*, and pAT-*hrpL* were constructed previously and their promoter activities were measured as described above (Yi et al., 2010).

4.4 | Pel activity assay and potato virulence assay

Dickeya Pel activity was measured by spectrometry as described in Matsumoto et al. (2003). In brief, an overnight culture of *Dickeya* in LB medium was transferred 1:100 to MM supplemented with 0.1% polygalacturonic acid (PGA) at 28°C and incubated with shaking for 16 h; 2 ml of bacterial culture was collected and centrifuged at 13,000 × *g* for 2 min, and the supernatant was transferred to a new Eppendorf tube for further analysis. To measure Pel activity, 10 μl of the supernatant was mixed with 990 μl of reaction buffer (0.05% PGA, 0.1 M Tris-HCl pH 8.5, 0.1 mM $CaCl_2$, prewarmed to 30°C). The optical density (OD) was measured at 230 nm for 3 min, and Pel activity was calculated by assuming that one unit of Pel activity results in an increase of 0.001 OD_{230} in 1 min. The potato maceration assay was performed using potatoes purchased from a local supermarket. Overnight bacterial culture (100 μl, OD_{590} = 1.0) was injected into surface-sterilized potatoes. The images were taken after 3 days of incubation at 28°C. Virulence was quantified by calculating the percentage of maceration area/total potato area using ImageJ software.

4.5 | Determination of the relative intracellular c-di-GMP concentration

We adopted a c-di-GMP responsive riboswitch method to determine relative intracellular c-di-GMP concentrations (Liang et al., 2020) where the *V. cholerae* Vc2 riboswitch (Sudarsan et al., 2008) from pRS414 was cloned into pRU1064 (a promoterless β-glucuronidase [GUS] reporter plasmid). The plasmid pRU1064-Vc2 was electrotransformed into *D. dadantii* 3937 and its derivatives, and the c-di-GMP levels were represented by the GUS activity. Overnight cell culture grown in MM (500 μl) was harvested by centrifugation at 10,000 × *g* for 3 min. The cell pellet was resuspended in PBS. After the addition of 50 μl 0.1% sodium dodecyl sulphate, 50 μl of chloroform was added. After the addition of the agent, the samples were vigorously vortexed. Samples were then centrifuged for 1 min at 10,000 × *g*. The upper fraction (100 μl) was collected and mixed with 890 μl PBS. Following the addition of 10 μl of 10 mM 4-methy lumbelliferyl-β-D-glucuronide (MUG; Sigma), 100 μl of the mixture was measured at an excitation wavelength of 365 nm and emission at 455 nm at 0, 5, 10, 15, and 20 min. The GUS value was standardized by conducting the Bradford assay (Bio-Rad) using 100 μl of the upper fraction from the GUS assay. Briefly, 700 μl water and 200 μl Bradford agent were mixed with 100 μl of the upper fraction from the GUS assay, and the absorbance at 595 nm was determined. Because c-di-GMP binds to the Vc2 riboswitch to suppress GUS expression, the relative GUS activity is inversely proportional to the cellular c-di-GMP concentration. Values were normalized to WT.

4.6 | Biofilm formation assay and swimming assay

The biofilm formation assay was conducted as described previously (Yi et al., 2010). In brief, *Dickeya* and its derivatives were streaked on LB agar plates, and single colonies of different strains were cultured in LB medium at 28°C overnight. Overnight culture was transferred 1:100 into MM in 1.5-ml Eppendorf tubes, and then 200 μl was

transferred into a 96-well microplate, which was incubated for 48 h. Crystal violet (1%) was used to stain the sessile cells for 15 min. The planktonic cells were removed by several gentle washes with water. After 5 h of air drying, the stained cells were dissolved in 90% ethanol and OD_{590} was determined. The swimming assay was conducted as described previously (Yuan et al., 2015). Cells were first grown in LB medium overnight at 28°C. The sample was then adjusted to OD_{590} = 1.0, and 10 μl overnight bacterial culture was inoculated in a swimming assay plate (MG plate containing 0.2% agar), which was incubated at 28°C. Swimming results are presented as the diameter of the radial growth area after 16 h.

4.7 | Statistical analysis

Statistical comparison of the data was performed using SPSS (IBM) and Excel (Microsoft). Data are presented as means ± standard error of the mean. Statistical significance ($p < 0.05$) was tested using one-way analysis of variance (ANOVA) with the post hoc Tukey's honestly significant difference test or Student's *t* test.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID

Manda Yu https://orcid.org/0000-0001-5932-9597
Ching-Hong Yang https://orcid.org/0000-0001-5330-7055

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SUPPORTING INFORMATION

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