The GacA-GacS Type Two-Component System Modulates the Pathogenicity of *Dickeya oryzae* EC1 Mainly by Regulating the Production of Zeamines

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The GacS-GacA type two-component system (TCS) positively regulates pathogenicity-related phenotypes in many plant pathogens. In addition, *Dickeya oryzae* EC1, the causative agent of soft rot disease, produces antibiotic-like toxins called zeamines as one of the major virulence factors that inhibit the germination of rice seeds. The present study identified a GacS-GacA type TCS, named TzpS-TzpA, that positively controls the virulence of EC1, mainly by regulating production of the toxin zeamines. RNA-seq analysis of strain EC1 and its *tzpA* mutant showed that the TCS regulated a wide range of virulence genes, especially those encoding zeamines. Protein-protein interaction was detected between TzpS and TzpA through the bacterial two-hybrid system and pull-down assay. In *trans* expression of *tzpA* failed to rescue the defective phenotypes in both the Δ *tzpS* and Δ *tzpS* Δ *tzpA* mutants. Furthermore, TzpA controls target gene expression by direct binding to DNA promoters that contain a Gac-box motif, including a regulatory RNA *rsmB* and the *vfm* quorum-sensing system regulator *vfmE*. These findings therefore suggested that the EC1 TzpS-TzpA TCS system mediates the pathogenicity of *Dickeya oryzae* EC1 mainly by regulating the production of zeamines.

Keywords: *Dickeya oryzae* EC1, GacA-GacS, regulation network, two-component system, zeamines

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The two-component system (TCS) signal transduction pathway is largely common in prokaryotes and a few eukaryotes such as yeasts, fungi, and protozoa (Beier and Gross 2006; García Vescovi et al. 2010; Mira-Rodado 2019; Tiwari et al. 2017). Notably, TCS allows bacteria to sense and respond to changes in specific chemical and physical parameters of their environment, including variations in nutrient levels, temperature, pH, autoinducer compounds, presence of antibiotics, osmolarity, and light (Brelund et al. 2017; Buschiazzi and Trajtenberg 2019; Collins and Childers 2021; García Vescovi et al. 2010). A typical TCS is composed of a membrane-bound sensor histidine kinase and a cytoplasmic response regulator. The transmembrane region of the sensor responds to environmental stimuli and perception. Once stimulated, the histidine residue of the histidine kinase domain is autophosphorylated by adenosine triphosphate (ATP). Briefly, after the autophosphorylation of the sensor kinase (SK) on a histidine residue, this sensor kinase transphosphorylates its cognate response regulator at a conserved aspartate residue located on its receiver domain, which potentially changes its ability to influence downstream gene expression, cell behavior, or enzyme activity. Finally, the phosphate group from the aspartate residue is transferred to water through a hydrolysis reaction (Buschiazzi and Trajtenberg 2019; Rajeev et al. 2020).

One notable TCS associated with the regulation of virulence in bacteria is the GacA-GacS type TCS, which was first described in the *Pseudomonas* genus as a global activator of antibiotic activity and ecological fitness (Laville et al. 1992). In this TCS, GacS belongs to the histidine sensor kinase that relays signals to the response regulator GacA (Heeb and Haas 2001). In addition, GacS-GacA TCS homologs with various regulatory roles have been identified in various plant and human pathogens, such as *Erwinia carotovora* subsp. *carotovora* (previously renamed *Pectobacterium wasabiae*), *Pseudomonas fluorescens*, *Salmonella enterica*, *Escherichia coli*, and *Vibrio cholerae* (Altier et al. 2000; Bull et al. 2001; Eriksson et al. 1998; Nagasawa et al. 1992; Wong et al. 1998). In the plant-pathogen *Pectobacterium wasabiae* SCC3193, the GacS-GacA homolog ExpS-ExpA controls the extracellular enzyme production (Eriksson et al. 1998). Moreover, the GacA homolog SirA in *S. enterica* serovar *Typhimurium* functions as a modulator of invasion as it positively regulates the invasive ability and type III secretion system (T3SS) along with its cognate sensor BarA (a GacS homolog) (Altier et al. 2000). Moreover, it was reported that mutations in either the *gacS* or *gacA* homologs of *E. coli* K-12 increased bacterial sensitivity to hydrogen peroxide, and this was attributed to *barA* given its

function in regulating adaptive responses in bacteria (Nagasawa et al. 1992). In *V. cholerae*, the GacA homolog, VarA, functions as a virulence associated regulator that positively modulates production of the cholera toxin and the toxin-coregulated pilus (Wong et al. 1998). Overall, the GacS-GacA TCS has two main functions, i.e., positive regulation of the biological control capacity of strains beneficial to plants and pathogenicity in plant or animal pathogenic bacteria (Heeb and Haas 2001).

The *Dickeya* spp. are among the most destructive plant pathogens, causing rot in several monocotyledons and dicotyledons, including rice, maize, potato, banana, and clivia (Hu et al. 2018; Wang et al. 2020; Zhou et al. 2011). In addition, several studies have revealed multiple virulence factors of the *Dickeya* genus. These include plant cell wall-degrading enzymes (PCWDEs) such as pectinases, cellulases (Cel), protease (Prt), xylanase, and their isozymes (Kotoujansky et al. 1985; Reverchon et al. 1985; van Gijsegem et al. 1985). Other identified virulence factors include extracellular polysaccharides (EPS) (Condemine et al. 1999), T3SS (Yap et al. 2005), siderophores (Franza et al. 2005), iron metabolism (Expert 1999), and the indigoidine pigment (Reverchon et al. 2002). Moreover, two structurally similar polyketide molecules, zeamine and zeamine II, identified in *D. oryzae* EC1 could strongly inhibit rice seed germination and had strong antibiotic activity against both gram-negative and gram-positive bacteria (Wu et al. 2010; Zhou et al. 2011). Purified zeamines efficiently repressed rice root germination more than shoot (Zhou et al. 2011). The phytotoxin was also shown to be highly active against some important pathogenic fungi (Cheng et al. 2013; Liao et al. 2015). Furthermore, some TCS proteins were shown to be involved in regulating the pathogenicity of bacteria in the *Dickeya* genus, especially in *D. dadantii* 3937. For example, the HrpX-HrpY TCS within the T3SS gene cluster was reported to play an essential role in regulating genes that are responsible for bacterial-plant interactions and bacterial aggregation (Yap et al. 2008). On the other hand, the VfmI-VfmH TCS within the *vfm* quorum sensing operon activates the transcription of PCWDE genes, biosynthesis of zeamines, as well as bacterial motility and aggregation (Lv et al. 2019; Nasser et al. 2013). Additionally, the PhoP-PhoQ TCS regulates the production of several virulence factors, such as pectate lyases (Pel), Cyt-like entomotoxins and iron metabolism (Costechareyre et al. 2012; Venkatesh et al. 2006). Notably, the GacS-GacA TCS in *D. dadantii* 3937 was shown to control the expression of T3SS, production of PCWDEs, formation of the pellicle, pathogenicity, and the systemic movement of the bacterium in *Saintpaulia ionantha* leaves (Lebeau et al. 2008; Yang et al. 2008). Additional studies also demonstrated that the GacS-GacA TCS mainly regulates these virulence factors through the secondary metabolite *rsmB*-RsmA pair, which is involved in posttranscriptional regulation, by binding to the messenger RNA (mRNA) of genes (Chatterjee et al. 2002; Liu et al. 1998; Yang et al. 2008). Consequently, the present study examined the function of the GacS-GacA homolog TzpS-TzpA, which positively mediates the virulence of *D. oryzae* EC1, mainly by regulating the toxin zeamines production rather than through the production of PCWDEs and T3SS.

RESULTS

The GacS-GacA homolog contributes to the pathogenesis of *D. oryzae* EC1.

The global GacS-GacA TCS has been reported to play a role in the pathogenicity of several gram-negative bacteria. Therefore, in order ascertain whether the GacS-GacA homolog in EC1 is required for virulence, the present study tested wild-type EC1 along with the $\Delta gacS$ and $\Delta gacA$ mutants, using the rice seedling model system. The results showed that the germination rate of rice seeds inoculated with $\Delta gacS$ and $\Delta gacA$ mutant

strains was increased to about 70 and 80%, respectively, compared with wild-type EC1, which completely inhibits germination of rice seeds, after one week (Fig. 1A; Supplementary Fig. S1A). Additionally, complementation of the $\Delta gacS$ and $\Delta gacA$ mutants with the corresponding wild-type genes restored the pathogenicity of the mutants to the wild-type level (Fig. 1A; Supplementary Fig. S1A). Additionally, the *D. oryzae* EC1 has been shown to be able to infect both monocotyledonous and dicotyledonous plants (Hu et al. 2018). Therefore, the virulence of *D. oryzae* EC1 in dicotyledons was assessed by testing the maceration of the $\Delta gacS$ and $\Delta gacA$ mutants in potato tubers. The results showed that deletion of either *gacS* or *gacA* led to smaller maceration zones on potato slices than those caused by wild-type EC1 and the complemented strains (Fig. 1B; Supplementary Fig. S1B). Quantitative analysis of the average length of the main root and shoot of rice seeds showed that, after treatment with $\Delta gacS$ and $\Delta gacA$ mutants for 1 week, both roots and shoots reached over 1.0 cm, a sharp contrast to the zero growth of seeds treated with wild-type EC1 and the complementary strains (Fig. 1C).

The GacS-GacA homolog primarily affects the production of zeamines.

Since both GacS and GacA were shown to positively control the virulence of *D. oryzae* EC1, the study then investigated which virulence factors could be regulated by this TCS system. In addition, given that zeamines are the main contributors to the pathogenicity of EC1 and the *zmsA* gene contributed to the production of zeamines (Zhou et al. 2011), the study first examined the secretion of these rice-specific phytotoxins in the $\Delta gacS$ and $\Delta gacA$ mutants. The results showed that deletion of either *gacS* or *gacA* led to a significant decrease in the production of zeamines at various cell densities, which is phenotypically close to the negative control $\Delta zmsA$ (Fig. 2). However, complementation of the $\Delta gacS$ and $\Delta gacA$ mutants through the respective *in trans* expression of *gacS* and *gacA*, totally restored the production of zeamines to a level similar to that in the wild-type strain (Fig. 2). Moreover, the GacS-GacA system was previously reported to modulate the production of PCWDEs and expression of T3SS in *D. dadantii* 3937 (Yang et al. 2008). The study therefore examined the production of Pel, Prt, and Cel in the $\Delta gacS$ and $\Delta gacA$ mutants. Surprisingly, the results revealed no significant differences between the wild type and mutant EC1 strains (Supplementary Fig. S1C). Expression of the three major regulatory genes for T3SS in the $\Delta gacA$ mutant was also comparable to that in the wild-type strain (Supplementary Fig. S2). Therefore, we renamed these two genes here as *tzpS* and *tzpA*, based on their function mainly regulating toxin zeamines production.

TzpS and TzpA regulate the motile and sessile phenotypes.

Previous research demonstrated the importance of motility and biofilms in the virulence of EC1, given that mutants with decreased motility or hyper-biofilms had an attenuated invasive ability (Chen et al. 2020; Shi et al. 2019). Herein, the results showed that deletion of *tzpS* and *tzpA* significantly reduced swimming motility to about 61.81 and 55.55% of the wild-type level, respectively (Fig. 3A). Furthermore, there was a decrease in the ability to form biofilms in both the $\Delta tzpS$ and $\Delta tzpA$ mutants (decreased to about half the wild-type EC1 level), as shown in Figure 3B. However, the ability of the $\Delta tzpS$ and $\Delta tzpA$ mutants to swim and form biofilms could be restored by *in trans* expression of *tzpS* and *tzpA*, respectively (Fig. 3).

TzpS and TzpA constitute a TCS that controls the expression of a variety of genes.

Numerous reports have demonstrated that GacS-GacA homologs were partners in a TCS that controlled multiple phenotypes

in various gram-negative bacteria (Heeb and Haas 2001; Pernestig et al. 2001; Workentine et al. 2009). In order to verify this hypothesis in EC1, the study used bacterial two-hybrid (B2H) system experiments to characterize interactions between the TzpS and

TzpA proteins, in which *tzpA* was ligated downstream of the RNA polymerase alpha subunit in the pTRG plasmid while *tzpS* was ligated downstream of the lambda repressor gene in the pBT plasmid. The recombinant plasmids were then co-introduced into

Fig. 1. Inhibition assay for EC1 and its derivatives in rice seed germination and the maceration test on potato tubers. **A**, Overnight bacterial cultures of wild-type EC1 along with the $\Delta gacS$ and $\Delta gacA$ mutants were adjusted to 10^7 CFU ml⁻¹. In addition, 15 rice seeds were treated with 10 ml of bacterial cultures, respectively, for 6 h, then, were washed three times with sterilized water and were incubated at 28°C. Water was added when necessary and the picture was taken 1 week after incubation. **B**, Overnight bacterial cultures of wild-type EC1 as well as the $\Delta gacS$ and $\Delta gacA$ mutants were adjusted to an optical density at 600 nm = 1.2, then, were spotted on the center of the potato tubers and were incubated at 28°C for 24 h until symptoms developed. **C**, Quantitative analysis of the effect of EC1 and the $\Delta gacS$ and $\Delta gacA$ mutants on root and shoot growth of rice seeds. The data are averages of at least 10 randomly selected seeds and error bars indicate standard deviation. Statistical analysis of the length of shoots and roots was performed separately, and the corresponding wild-type EC1 values were used as controls. ns indicates $P > 0.05$, one asterisk (*) indicates $P < 0.05$ and three (***) $P < 0.001$, as determined by one-way analysis of variance with multiple comparison test.

Fig. 2. Analysis of zeamines production by wild-type EC1 and its derivatives. Statistical analysis of zeamines production in wild-type EC1, the $\Delta gacS$ and $\Delta gacA$ mutants, and its complementary strains at three different cell densities. The dotted line indicates the wild-type levels of antibiotic production. The experiments were performed in triplicate and the result of one representative experiment is presented. The error bars represent standard deviations. Statistical analysis of zeamines production units was performed within each optical density (OD) panel, and the corresponding wild-type values were used as controls. Statistical significance was determined using the Holm-Sidak method of multiple t test, with alpha (adjusted P value) = 0.01. Two asterisks (**) indicate $P < 0.01$, ns = not significant, $P > 0.01$.

the reporter strain XL1-Blue MRF'. The results in Figure 4A show that the reporter strain harboring the plasmid pair pBT-TzpS/pTRG-TzpA could grow on the selective medium plate containing 5 mM 3-amino-1,2,4-triazole (3-AT), although the clones were scarcer than those produced on the plate with the positive-control pair pBT-LGF2/pTRG-Gal11P. In agreement with this observation, pull-down results demonstrated a binding of TzpA with TzpS (Fig. 4B and C). In addition, complementary expression of *tzpA* in Δ *tzpS* and the double mutant Δ *tzpS* Δ *tzpA* could not restore both swimming and the ability to produce zeamines to the level of wild-type EC1 (Fig. 4D and E). To determine the comprehensive regulatory role of TzpS-TzpA TCS in bacteria, the present study compared and analyzed the transcriptomes of the deleted mutant Δ *tzpA* and wild-type EC1 through RNA-seq analysis. Strains were grown in Luria Bertani (LB) medium and were harvested during the midlog growth phase (optical density at 600 nm [OD₆₀₀] = 0.6). RNA extraction and transcriptome sequencing were carried out as described below. The differentially expressed genes (DEGs) results showed that 108 genes were significantly upregulated, while 85 were downregulated (fold change ≥ 2.0) in the Δ *tzpA* mutant, as compared with the wild-type EC1 (Supplementary Table S1). In addition, these DEGs were shown to be involved in multiple biological processes, including the bacterial secretion system, TCS, transporters, virulence, membrane components, and signal transduction (Supplementary Table S1). More specifically, the expression levels of half of the *zms* genes were reduced more than twofold (Supplementary Table S1), consistent with the phenotypic results from the zeamines bioassay (Fig. 2). Moreover, the RNA-seq results were validated through quantitative PCR (qPCR) analysis of some selected DEGs (Supplementary Table S1).

The REC (CheY-homologous receiver) domain of TzpA is important for its biological function.

The TzpS protein has six domains, namely, a domain of unknown function, HAMP (histidine kinases, adenyl cyclases, methyl binding proteins, phosphatases) domain, His kinase A domain, histidine kinase-like ATPase domain, the REC domain, and the HPT (histidine phosphotransfer) domain. Additionally, the TzpA protein domain comprises of a REC domain and a helix-turn-helix (LuxR-type) domain (Fig. 5A). In order to characterize

the conserved aspartate residues in the REC domain of TzpA that participated in receiving phosphate groups from TzpS, the study used a multiple protein sequence alignment tool to compare the REC domain of TzpA to 10 other canonical REC domains. The alignment results revealed that two conserved aspartate residues (D49 and D54), located at positions 49 and 54 of the TzpA protein, were potentially important for receiving the phosphoryl group (Supplementary Fig. S3). Consequently, several *tzpA* derivatives in Δ *tzpA* were generated, including in *trans* expression of *tzpA* without the REC region and with single-residue mutations (D49A and D54A), respectively, in order to verify the function of these two aspartic acid residues. The results showed that overexpression of the intact *tzpA* in the Δ *tzpA* mutant restored both zeamines production and biofilm formation, while complementary expression of the REC and D49A mutated *tzpA* failed to completely recover these phenotypes (Fig. 5B and C). However, overexpression of the D54A residue mutated *tzpA* in Δ *tzpA* could fully restore the phenotypes (Fig. 5B and C).

The TzpS-TzpA system modulates small RNA (sRNA) *rsmB* transcription by binding to the Gac-box motif.

A previous study on *D. dadantii* 3937 reported that one of the downstream regulators of the GacS-GacA TCS was a regulator of the secondary metabolites, a RsmA-*rsmB* pair (Lapouge et al. 2008; Yang et al. 2008). The study showed that GacA positively regulates *rsmB* by directly binding to the Gac-box in its promoter region (Fig. 6A). Consequently, the present study conducted the gene reporter experiment by fusing the promoter region of *rsmB* upstream of the coding region of GFP (green fluorescent protein), in order to confirm the binding sites of TzpA in the *rsmB* promoter region. The results revealed that there was a sharp decrease in the expression levels of *rsmB* in both the Δ *tzpA* and Δ *tzpS* mutants compared with wild-type EC1 (Fig. 6B and C). Similarly, replacement of the original Gac-box sequence, respectively, with AACCAGAGATCTCTTAC and TGTACCTTTTCTCTTACA (named *prsmBMA*-GFP and *prsmBMB*-GFP, respectively) led to a decrease of about 80% in the expression of *rsmB* (Fig. 6C). In agreement with this observation, electrophoretic mobility shift analyses (EMSA) demonstrated TzpA binding to *rsmB* promoter in vitro (Fig. 6D).

Fig. 3. Motile and sessile phenotypes of wild-type EC1 (WT) as well as the Δ *tzpS* and Δ *tzpA* mutants. **A**, Swimming motility. **B**, Biofilm formation. The dotted lines indicate the WT level. Four asterisks (****) indicate $P < 0.0001$, three (***) $P < 0.001$ and ns = not significant, $P > 0.01$ (the P values were obtained from the Student's t test). Each experiment was done in triplicate with at least two independent repeats.

Fig. 4. TzpS and TzpA constitute a two-component system. **A**, Analysis of the interaction between TzpS and TzpA through the bacterial two-hybrid (B2H) assay. The B2H reporter strain XL-1 Blue MRF⁺ was cotransformed with different plasmid pairs and, then, applied on nonselective (left panel) and selective (right panel, containing 5 mM 3-amino-1,2,4-triazole) screening media, respectively. The pBT-LGF2 and pTRG-GAL11P pair was used as a positive control. Glutathione-S-transferase (GST) pull-down experiment analysis of interaction between TzpS and TzpA in vitro. **B**, Western blot analysis of the purified TzpA and TzpS proteins. **C**, GST pull-down experiment with lysates mixtures from cells expressing TzpA-His and from cells expressing either GST or GST-TzpS. After pull-down, protein complexes were eluted with 10 mM reduced glutathione. The interaction was analyzed by immunoblot with anti-His and anti-GST. In *trans* expression of *tzpA* could not restore the deficient phenotypes of the Δ *tzpS* mutant and the Δ *tzpS* Δ *tzpA* double mutant to those of the wild-type level. **D**, Swimming motility and **E**, zeamines production analyses. The dotted line indicates the wild-type level. For statistical comparisons, ordinary one-way analysis of variance with Tukey's multiple comparisons test was used. Different lower-case letters represent statistically significant differences. For D, $P < 0.001$, with two or more independent experiments performed in triplicate; for E, $P < 0.05$, with three or more independent experiments performed in triplicate.

DISCUSSION

TCS is a major strategy used by microbes to control their gene expression profiles in response to various intracellular and extracellular environmental stimuli. In addition, previous studies demonstrated the importance of the GacS-GacA type TCS in regulating the virulence of plant and animal pathogens. In this study, the GacS-GacA type TCS homolog TzpS-TzpA was examined in *D. oryzae* EC1. TzpS-TzpA mainly regulates the production of toxic zeamines, cell motility, and biofilm formation at the transcriptional level (Fig. 7). Although the GacS-GacA type TCS regulates the pathogenicity of both *D. oryzae* EC1 and *D. dadantii* 3937, the present study interestingly uncovered that this TCS positively regulates the production of zeamines (Fig. 2) but not the production of PWCDEs and the expression of T3SS genes in EC1 (Supplementary Figs. S1 and S2). However, deletion of either *gacS* or *gacA* in *D. dadantii* 3937 significantly reduced the production of PWCDEs and expression of T3SS (Lebeau et al. 2008; Yang et al. 2008). These results may be explained by the fact that the regulatory networks of the GacS-GacA type TCS are different between these two bacteria. The different findings may also have been due to the distinct determinants of virulence between the two bacteria. In addition, RNA-seq and qPCR analysis showed expression levels of more than half the genes within the *zms* cluster of Δ *tzpA* mutant reduced more than twofold compared with wild-type EC1 (Supplementary Fig. S4A). EMSA assay did not reveal any band shift between TzpA protein and the

promoter of either the *zmsA* or *zmsK* gene, suggesting that TzpA protein does not directly regulate the zeamine biosynthesis (Supplementary Fig. S4B). A previous study by our research group also showed that several transcription factors controlled the production of zeamines and were regulated by the SlyA-Fis-*zms* or SlyA-Fis-VfmE-*zms* gene pathways (Lv et al. 2018; Zhou et al. 2016). Herein, transcriptome analysis showed that TzpA regulates the expression of *vfmE* (Supplementary Table S1). Moreover, a Gac-box binding site (−7 bp TGACACCATGAGCTTACA +11 bp) was identified in the *vfmE* promoter and open reading frame (ORF) region but not in *zmsA* and *zmsK* genes, and an EMSA assay confirmed TzpA binding to this region (Supplementary Fig. S4C), suggesting that TzpA might regulate the biosynthesis of zeamines through VfmE and the Gac-box binding site is important for this function. However, the specific relationship among these regulators is still unclear and should therefore be explored further.

The study also found that deletion of either *tzpS* or *tzpA* decreased swimming motility as well as biofilm formation in *D. oryzae* EC1 (Fig. 3). Previous studies by our research group highlighted the importance of a bacterial second messenger, c-di-GMP, and the putrescine signal in controlling the motile and sessile phenotypes (Chen et al. 2020; Shi et al. 2019). In the present study, the expression level of a few genes associated with c-di-GMP metabolism and putrescine transport changed more than twofold in the Δ *tzpA* mutant (Supplementary Table S1). In addition, the results showed that deletion of *tzpS* and *tzpA* had no

Fig. 5. The REC domain of TzpA plays a role in signal sensing. **A**, The predicted domain architectures and consensus sequence motifs of TzpS and TzpA in *Dickeya oryzae* EC1. DUF = domain of unknown function, HAMP = histidine kinases, adenyl cyclases, methyl binding proteins, phosphatases domain, HisKA = his kinase A (phosphoacceptor) domain, HATPase_c = histidine kinase-like ATPase domain, REC, receiver domain, HPT = histidine phosphotransfer domain, HTH = helix-turn-helix DNA-binding domain. **B**, Effects of TzpA variants, TzpA Δ REC, TzpA Δ D49A, and TzpA Δ D54A on zeamines production and **C**, biofilm formation. Dotted lines indicate the wild-type level. Four asterisks (****) indicate $P < 0.0001$, three (***) $P < 0.001$, two (**) $P < 0.01$, one (*) $P < 0.05$, and ns = not significant, $P > 0.05$ (the P values were obtained from Student's t test, with two or more independent experiments performed in triplicate).

effect on the concentrations of c-di-GMP and putrescine (Supplementary Fig. S5). These findings, therefore, suggested the presence of a signaling mechanism in which the TzpS-TzpA TCS controls motility and biofilm formation, independent of the c-di-GMP and putrescine signaling pathways (Fig. 7). However, it is still unclear how these three pathways coordinate to regulate bacterial motility and biofilm phenotypes.

The above results indicated that Δtzs and Δtpa presented the similar phenotypes in all tested experiments. Due to the specificity of the transfer process of phosphate groups and the associated receiver proteins in the TCS, the two proteins in the typical TCS usually regulate the same phenotype. In this study, the B2H system and in vitro pull-down assay were used to confirm that TzpS-TzpA is a TCS in *D. oryzae* EC1 (Fig. 4). Complementary expression of the *tpa* gene in the Δtzs and $\Delta tzs\Delta tpa$ mutants failed to restore the motility and zeamine-production phenotypes, respectively (Fig. 4D and E), which means that the functionality of TzpA is dependent on the presence of TzpS. Additionally, transcriptome profiling by RNA-seq identified significant changes in the expression of nearly 200 genes in the Δtpa mutant compared with wild-type EC1 (Supplementary Table S1). In summary, these results confirmed that TzpS and TzpA form a TCS that regulates many biological functions in *D. oryzae* EC1.

Notably, the typical signal transduction model of the GacS-GacA TCS consists of a conserved four-step phosphorelay mechanism that includes autophosphorylation at the first histidine residue (H1), after which the phosphoryl group is transferred to the aspartate (D1), then to the histidine (H2) residue of GacS, and, finally, to a conserved aspartate (D2) in the REC domain of GacA (Heeb and Haas 2001; Latour 2020; Workentine et al. 2009). In the present study, analysis of the TzpS protein domains showed that the HisKA, REC, and HPT domains contain H1, D1, and H2,

respectively while the REC domain of TzpA contains D2 (Fig. 5A). In addition, in *trans* expression of the REC-deleted TzpA in the Δtpa mutant failed to restore zeamine-production properties and biofilm formation in the bacteria (Fig. 5B and C), highlighting the importance of the REC domain in the signal transduction of the TzpA response regulator. Moreover, the complemented strain could not recover the defective phenotype of the Δtpa mutant after replacing the aspartate residue with alanine in position 49 but not 54 of the REC domain of TzpA (Fig. 5B and C). Moreover, phenotypic experiments indicated that all three mutants of Δtzs (Δtzs^{H299A} , Δtzs^{D722A} , and Δtzs^{H826A}) also reduced production of zeamines and failed to inhibit seed germination, consistent with phenotypes of Δtzs mutant (unpublished data). These results indicated that domains and residues predicted on the phosphorylation of TzpS-TzpA TCS play a key role in its biological functions. Nevertheless, the precise phosphorylation and transphosphorylation processes of the TzpS-TzpA TCS and the exact mechanisms used by TzpS to sense and transduce environmental signals remain unclear and should be studied in future research.

In addition, the regulatory role of the GacS-GacA type TCS is usually exerted through the regulator of the Rsm system, which includes untranslated sRNAs and sRNA-binding proteins (Lapouge et al. 2008). In *D. dadantii* 3937, *rsmB* (a small untranslated RNA) is transcriptionally regulated by GacA, which binds to a posttranscriptional regulatory protein, RsmA, to neutralize its negative regulatory effect (Yang et al. 2008; Yuan et al. 2019). The results from the current study confirmed that TzpS-TzpA activates the transcription of *rsmB* in *D. oryzae* EC1 (Fig. 6B and C), and an EMSA assay confirmed that the TzpA protein bound to the promoter region of *rsmB* in vitro (Fig. 6D). Moreover, the phosphorylated GacA protein was reported to bind to a conserved motif

Fig. 6. TzpA binds to the Gac-box in the promoter region of *rsmB*. **A**, Sequence analysis of the promoter region of *rsmB* using CLC Main Workbench. The sequences of the open reading frame region of *rsmB* are marked with a yellow arrow. The binding site of GacA, determined in this study, is highlighted with a green arrow. **B**, Expression of *rsmB* in wild-type EC1 (blue dots) and the *tpzS* mutant (red squares), grown in Luria Bertani medium. **C**, Expression of *rsmB* and its derivatives in wild-type EC1 (red squares for *rsmB*, green triangles for *rsmBMA*, blue triangles for *rsmBMB*) and the *tpzA* mutant (orange diamonds). Promoter activities were measured up to 30 h, and the fluorescence values were obtained every 2 h at an excitation wavelength of 485 nm and an emission wavelength of 528 nm. Each experiment was done in triplicate with at least two independent repeats. **D**, Electrophoretic mobility shift detection of TzpA binding to the promoter DNA of *rsmB* with increasing concentrations of TzpA.

upstream of the *rsmB* gene promoter, known as the Gac-box (consensus TGTAAGN₆CTTACA, where N is any nucleotide). As expected, one Gac-box was identified upstream of the ORF of *rsmB* (Fig. 6A). Furthermore, the results revealed that replacement of the Gac-box sequence blocked the transcriptional activation of *rsmB* (Fig. 6C). These findings suggested that the expression of *rsmB* is upregulated by GacS-GacA TCS through the Gac-box in EC1; further study would be needed to determine the specificity of binding between TzpA and *rsmB*.

Overall, the present study demonstrated that the GacS-GacA type TCS TzpS-TzpA plays an essential role in regulating biological functions and virulence in *D. oryzae* EC1, especially in controlling the production of toxic zeamines. The results also revealed that the GacS-GacA type TCS is conserved in the regulation of pathogenicity in EC1 and other plant pathogens, providing a new target for designing inhibitors of this key signaling system.

MATERIALS AND METHODS

Bacterial strains and growth conditions.

The bacterial strains and plasmids used in this study are described in Supplementary Table S2. Briefly, *E. coli* was routinely grown at 37°C in LB medium. Cultures of *D. oryzae* EC1 and its derivative strains were established in LB medium, at 28°C. All the conjugation experiments were performed using minimal medium agar plates (Hussain et al. 2008). In addition, the LS5 medium was used for measuring the production of zeamines, as previously reported (Chen et al. 2016). Moreover, antibiotics were added accordingly, at the following final concentrations: streptomycin, 50 µg/ml; ampicillin, 100 µg/ml; kanamycin, 50 µg/ml; and polymyxin, 30 µg/ml. Bacterial growth was also assessed by measuring the OD₆₀₀ of the growth medium, using the NanoDrop 2000c (Thermo Fisher Scientific, Shanghai, China).

Fig. 7. A Schematic diagram of the regulatory network of the TzpS-TzpA two-component system (TCS) in *Dickeya oryzae* EC1. TzpS is involved in the perception of unknown environmental signals and phosphorylates TzpA, which activates the function of TzpA, and the TCS positively controls the expression of the *rsmB* gene. In addition, the TzpS-TzpA TCS regulates a wide range of physiological functions, including biofilm formation, swimming motility and production of toxic zeamines. The putrescine signaling pathway positively regulates biofilm formation and motility, which is independent of TzpS-TzpA. Solid arrows indicate direct signal regulation, while the dashed arrow indicates indirect regulation.

Construction of mutants and in trans complementation.

The mutant construction assay was performed using the suicide vector pKNG101 and triparental mating to generate gene in-frame deletion mutants, following a previously reported protocol (Chen et al. 2016). Additionally, the coding regions of *tzpS*, *tzpA*, and *rsmB* were amplified through PCR, using the gene-specific flanking primers listed in Supplementary Table S3. Moreover, in trans complementation was conducted by constructing the pBBR1MCS4-*tzpS* and pBBR1MCS4-*tzpA* plasmids to express the *tzpS* and *tzpA* genes, respectively using their native promoters. The coding regions of the target genes were then amplified through PCR, using the specific primers listed in Supplementary Table S3.

Residue-directed mutagenesis.

Residue mutations in pBBR1-MCS4-*tzpA* were generated using the Mut Express II fast mutagenesis kit V2 (Vazyme Biotech, Nanjing, China). Briefly, in order to introduce the desired point mutations, pBBR1-MCS4-*tzpA*^{D49A} and pBBR1-MCS4-*tzpA*^{D54A} plasmids were amplified using the Phanta Max super-fidelity DNA polymerase (Vazyme Biotech) and primer pairs containing the desired nucleotide substitutions (Supplementary Table S3). The reaction mix was then digested with the *DpnI* restriction enzyme and was transformed into DH5α. Finally, mutations were confirmed through sequencing.

Construction of the transcriptional fusion plasmid and assessment of fluorescence.

The 322-bp DNA fragment upstream of the ORF of *rsmB* was amplified using the *prsmB*-GFP-F and *prsmB*-GFP-R primers listed in Supplementary Table S3, then was fused into the promoterless *gfp*-reporter plasmid pPROBE-NT (Miller et al. 2000), to generate the *prsmB*-GFP construct. In addition, *prsmB*-GFP and pNT (blank control) were separately conjugated into the wild-type EC1 and Δ *tzpA* mutant through triparental mating. Moreover, mutated plasmids were constructed by introducing a mutation into the conserved Gac-box region using the Mut Express MultiS fast mutagenesis kit V2 (Vazyme Biotech). The pNT-*rsmB*-Mut-A1/A2 and pNT-*rsmB*-Mut-B1/B2 primer pairs were also used to generate EC1 (*prsmB*_{MA}-GFP) and EC1 (*prsmB*_{MB}-GFP).

Additionally, fluorescence measurements were taken using 96-well polypropylene microtiter dishes. Briefly, overnight cultures of bacteria grown in LB broth were adjusted to the same optical density and were diluted 100-fold in the same medium. Thereafter, 100 µl of the inoculated culture was grown in each well at 28°C, while shaking at 200 rpm for up to 30 h. The green fluorescence intensity (excitation/emission = 485/528 nm) of each well was then measured every 2 h, using a microplate reader (Syngery H1; BioTek, Winooski, VT, U.S.A.).

Swimming motility assay.

Collective swimming motility was assessed on LB plus 0.3% (wt/vol) agar medium plates. Briefly, overnight bacterial cultures were adjusted to the same optical density. Thereafter, 1 µl of each sample was spotted onto the center of the plate, which was then incubated at 28°C for 18 to 24 h.

Biofilm formation assay.

The biofilm formation assay was conducted according to a previously described protocol (Chen et al. 2020). In brief, overnight bacterial cultures were diluted, at a ratio of 1:1,000 with super optimal broth medium plus 1 % sucrose in glass test tubes, and then incubated at 28°C without shaking. After 24 h, the biofilm mass was washed with tap water, then was stained with 0.1% crystal violet (wt/vol) for 10 to 15 min. Afterward, the unbound dye was removed by rinsing three times with tap water.

The stained biofilm mass was then decolorized, using 70% ethanol, and was quantified at an absorbance of 570 nm in a microplate reader (Synergy H1).

The zeamines production assay.

The zeamines production assay was carried out in the LS5 medium and *E. coli* DH5 α was used as the indicator bacterium, as previously described (Chen et al. 2016). Zeamines (units) = $0.5484^{e^{0.886x}}$ ($R^2 = 0.9957$), x is the width in millimeters of the growth inhibition zone surrounding each well.

The PCWDE assay.

The PCWDE enzymatic activities were measured in a specific medium, as previously described but with minor modifications (Lv et al. 2019). Briefly, 30 ml of the assay medium was poured into a 10 $\times$ 10 cm square plate, then, wells of 5 mm in diameter were punched out after solidification. In order to test the production of PCWDEs, bacterial cells were grown to the logarithmic phase, then adjusted to OD₆₀₀ = 1.5. Each sample (20 μ l) was then added to the wells and the plate was incubated at 28°C. After 13 h, the Pel assay plate was prepared with 1 M HCl, while the Cel assay plate was colored with 0.1% Congo Red (wt/vol) for 15 min, then was discolored using 1 M NaCl. After 24 h, halos around the wells became visible in the Prt assay plate without any further treatment.

The B2H assay.

The B2H assay was conducted using the BacterioMatch II two-hybrid system kit (Stratagene, San Diego, CA, U.S.A.), in order to assess the presence of protein-protein interactions between TzpS and TzpA. In this assay, approximately 50 μ g of each plasmid harboring pTRG-*tzpA* and pBT-*tzpS* were cotransformed into the host strain XL1-Blue MRF⁺ Kan through chemical transformation after adding 1 ml of preheated LB medium in each tube. The cells were then incubated at 37°C while shaking at 200 rpm for 2 h. Thereafter, the LB medium was removed through centrifugation, then the cells were resuspended in 1 ml of M9+His-dropout broth and were incubated at 37°C, while shaking at 200 rpm for another 2 h. Cotransformation mixtures were then grown on nonselective (M9 + His-deficient [NSM]) and selective (M9 + His-deficient + 5 mM 3-AT [SSM]) screening media for 24 to 36 h at 37°C. Notably, incubation was conducted for an additional 24 to 36 h at 30°C in the dark, for the weak interactors. Colonies that grew on both the NSM and SSM plates were identified as positive interaction pairs. However, colonies that were observed only on NSM but not on SSM indicated that there was no interaction between the proteins.

Pathogenicity tests.

In order to conduct the rice seed germination assay, the rice cultivar CO39 was used in the pathogenicity tests. Briefly, bacterial cells were collected and resuspended in phosphate buffered saline (PBS) buffer and, then, adjusted to 10⁷ CFU ml⁻¹. Fifteen rice seeds were then immersed in 10 ml of each bacterial dilution culture for 6 h at room temperature, after which they were washed with sterilized water and were transferred onto moistened filter papers in a Petri dish. Each dish was incubated at 28°C under 16-h-light and 8-h-dark cycles for up to 1 week. The same amount of PBS buffer was used as a blank control. Afterward, the rate of seed germination and the length of rice root and shoot were evaluated at the indicated timepoints. In order to conduct the potato tubers maceration test, a potato (*Solanum tuberosum*) was washed with tap water, was dried on a tissue paper, was cut into uniform slices with a thickness of about 5 mm, and were washed three times with sterilized water. The slices were then dried on a sterilized filter paper, after which they were transferred onto dishes containing three wet filter papers. Thereafter, 2 μ l of bacterial

culture (OD₆₀₀ = 1.2) was spotted on the center of the slices, which were then incubated at 28°C for 24 h until symptoms developed. The experiment was repeated two times in triplicate.

RNA extraction and real-time qPCR assays.

RNA extraction was conducted using the TRIzol reagent and the Phasemaker Tubes complete system (Thermo Fisher Scientific), as previously described (Chen et al. 2020). Thereafter, the TransScript one-step gDNA removal and cDNA synthesis SuperMix (TransGen Biotech, Beijing) was used to synthesize the cDNA for each RNA sample. In addition, the qPCR assay was performed, using the Talent qPCR PreMix (SYBR Green) (Tiangen Biotech, Beijing) and the Applied Biosystems QuantStudio 6 Flex real-time PCR system (Thermo Fisher Scientific), according to manufacturer protocol. The internal reference gene was *gapA* and the relative level of gene expression was calculated using the $\Delta\Delta C_T$ method, according to the MIQE guidelines (Bustin et al. 2009; Taylor et al. 2010). The primers for the SYBR green qPCR were designed using the Beacon designer software and are listed in Supplementary Table S3.

RNA-seq transcriptome analyses.

The RNA-seq transcriptome analyses were conducted using a previously described method but with minor modifications (Lv et al. 2019). Briefly, RNA sequencing was performed on two strains, i.e., the wild-type EC1 and Δ *tzpA* mutant, using an Illumina HiSeq 2500 sequencer from the Gene Denovo Biotechnology Co. (Guangzhou, China). In addition, total RNA was extracted from the samples and mRNA was enriched after removing ribosomal RNA, using a Ribo-Zero magnetic kit (Illumina, San Diego, CA, U.S.A.). Thereafter, the mRNA was fragmented into short fragments, then reverse-transcribed into cDNA, according to the manufacturer protocol. The cDNA fragment was then purified and ligated to sequencing adapters before sequencing.

After sequencing, clean reads were obtained by removing adaptors, unknown nucleotides, and low-quality reads. The clean reads were then aligned to the reference genome of *D. oryzae* EC1, using the TopHat2 version 2.0.3.12 software (Kim et al. 2013). Additionally, the FPKM (fragments per kilobase of transcript per million mapped reads) method was used to normalize the level of gene expression using the following formula (Trapnell et al. 2012):

$$FPKM = \frac{10^6 C}{NL/10^3}$$

The calculated level of gene expression was then directly used to assess differences in gene expression among the samples.

Moreover, the edgeR package was used to identify the DEGs across the samples. Notably, the significant DEGs were identified using a fold change ≥ 2 and a false discovery rate < 0.05 . The DEGs were then subjected to enrichment analysis through gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses. Additionally, GO-TermFinder was used to identify GO terms that annotated a list of enriched genes with a significant P value < 0.05 , through the following formula:

$$P = 1 - \sum_{i=0}^{m-1} \frac{\binom{M}{i} \binom{N-M}{n-i}}{\binom{N}{n}}$$

On the other hand, KEGG was used to highlight the enriched metabolic or transduction pathways in DEGs, compared with the whole genome (Kanehisa et al. 2008). The same formula as that used in GO analysis was employed.

Protein expression and purification.

The coding regions of TzpS and TzpA were amplified with the primers listed in Supplementary Table S3 and were fused to the expression vectors pGEX-6p-1 and pET-32a, respectively. The fusion gene constructs were transformed into *E. coli* BL21 (DE3) pLysS. Affinity purification of glutathione-S-transferase (GST)-TzpS and TzpA-His fusion protein was performed using Glutathione-Superflow Resin and the HisTALON gravity column purification kit, according to the operating instructions (Takara, Beijing, China). The purified fusion proteins were analyzed by Western blot using anti-GST and anti-His monoclonal antibody (Abbkine, Wuhan, China), respectively.

Gel EMSA.

The DNA probes used for EMSA were prepared by PCR amplification using the primer pairs listed in Supplementary Table S3. The purified PCR products were 3'-end labeled with biotin following manufacturer instructions (Thermo Fisher Scientific). The DNA-protein binding reactions were performed according to the manufacturer instructions for the LightShift chemiluminescent EMSA kit (Thermo Fisher Scientific). A 6% polyacrylamide gel was used to separate the DNA-protein complexes. After UV cross-linking, the biotin-labeled probes were detected in the positively charged nylon transfer membrane (Merck KGaA, Darmstadt, Germany), using a chemiluminescence system (Tanon, Shanghai, China).

In vitro pull-down assay.

Pull-down assay was conducted following the manufacturer instructions for the Pierce GST protein interaction pull-down kit (Thermo Fisher Scientific). In brief, GST-TzpS was used as bait, and TzpA-His was used as prey. For the binding assays, 150 µg of GST-RqpS was immobilized into 50 µl glutathione agarose resin, then the same amount of prey protein TzpA-His was added to the same column for capture. After being washed with wash solution, protein complexes were eluted with 10 mM reduced glutathione and were analyzed by Western blot, using anti-GST and anti-His monoclonal antibody (Abbkine), respectively.

Bacterial growth curve analysis.

Overnight bacterial cultures were adjusted to the same optical density then diluted 100-fold using the LB medium. Thereafter, 200 µl of the diluted culture was inoculated, was then grown in each well of a 96-well plate at 28°C, and was incubated, with low intensity shaking, on the Synergy H1 multilabel reader (BioTek).

The *D. oryzae* EC1 genome sequence can be accessed from the National Center for Biotechnology Information (NCBI), under accession number NZ_CP006929.1. The raw data of transcriptome analyses were available from the Sequence Read Archive (SRA) under accession number PRJNA726287.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

Beacon website: http://www.premierbiosoft.com/molecular_beacons

NCBI *D. oryzae* EC1 genome sequence:

https://www.ncbi.nlm.nih.gov/nuccore/NZ_CP006929.1

SRA raw data: <https://dataview.ncbi.nlm.nih.gov/object/PRJNA726287>

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