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e-**X**tra*

The Degenerate EAL-GGDEF Domain Protein Filp Functions as a Cyclic di-GMP Receptor and Specifically Interacts with the PilZ-Domain Protein PXO_02715 to Regulate Virulence in *Xanthomonas oryzae* pv. *oryzae*

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Degenerate GGDEF and EAL domain proteins represent major types of cyclic diguanylic acid (c-di-GMP) receptors in pathogenic bacteria. Here, we characterized a FimX-like protein (Filp) which possesses both GGDEF and EAL domains in *Xanthomonas oryzae* pv. *oryzae*, the causal agent of bacterial blight of rice. Both in silico analysis and enzyme assays indicated that the GGDEF and EAL domains of Filp were degenerate and enzymatically inactive. However, Filp bound to c-di-GMP efficiently within the EAL domain, where Q^{477}, E^{653}, and F^{654} residues were crucial for the binding. Deletion of the *filp* gene in *X. oryzae* pv. *oryzae* resulted in attenuated virulence in rice and reduced type III secretion system (T3SS) gene expression. Complementation analysis with different truncated proteins indicated that REC, PAS, and EAL domains but not the GGDEF domain were required for the full activity of Filp in vivo. In addition, a PilZ-domain protein (PXO_02715) was identified as a Filp interactor by yeast two-hybrid and glutathione-*S*-transferase pull-down assays. Deletion of the *PXO_02715* gene demonstrated changes in bacterial virulence and T3SS gene expression similar to Δ*filp*. Moreover, both mutants were impaired in their ability to induce hypersensitive response in nonhost plants. Thus, we concluded that Filp was a novel c-di-GMP receptor of *X. oryzae* pv. *oryzae*, and its function to regulate bacterial virulence expression might be via the interaction with PXO_02715.

Cyclic diguanylic acid (c-di-GMP), a ubiquitous bacterial second messenger, has been implicated in regulation of various cellular behaviors such as the lifestyle transition between motile and sessile states (Hengge 2009; Romling et al. 2013). It also controls the virulence of pathogenic bacteria by regulating the production of exopolysaccharides (EPS) and extracellular enzymes, the expression of type III secretion system genes, and so on (Fouhy et al. 2006; Kalia et al. 2013; Kulasakara et al. 2006; Yi et al. 2010). Synthesis and degradation of c-di-GMP is controlled by the opposing activities of diguanylate cyclases (DGC), which contain the GGDEF domain, and phosphodiesterases (PDE), which harbor EAL or HD-GYP domains (Schirmer and Jenal 2009). Genomic studies have revealed that a large number of these domains exist in different bacterial species (Seshasayee et al. 2010).

Identification and functional characterization of c-di-GMP receptors have indicated that the downstream signaling mechanisms of the c-di-GMP pathway could be versatile. PilZ domains are the first c-di-GMP-binding modules identified through bioinformatics analysis, and they are widely distributed in bacterial genomes (Amikam and Galperin 2006). Thus far, a few of them have been experimentally confirmed (Merighi et al. 2007; Pratt et al. 2007; Pultz et al. 2012; Ryjenkov et al. 2005; Zorraquino et al. 2013). For example, YcgR from *Escherichia coli* was shown to regulate swimming speed by directly interacting with flagellar motor components (Boehm et al. 2010; Paul et al. 2010). Transcriptional regulators FleQ from *Pseudomonas aeruginosa* (Hickman and Harwood 2008) and CRP-like protein (Clp) from *Xanthomonas campestris* pv. *campestris* and *X. axonopodis* pv. *citri* were also found to be c-di-GMP-binding proteins (Leduc and Roberts 2009; Tao et al. 2010). In addition, some degenerate GGDEF or EAL domains which do not function as DGC or PDE can still perform biological functions as c-di-GMP receptors, such as FimX, LapD, and SgmT (Navarro et al. 2009; Newell et al. 2009; Petters et al. 2012; Schirmer and Jenal 2009). Moreover, mRNA riboswitches that specifically bind to c-di-GMP have also been identified (Lee et al. 2010). These findings indicate that c-di-GMP signaling interferes with cellular processes at various levels, including gene transcription, translation, and post-translation.

X. oryzae pv. *oryzae* causes bacterial leaf blight of rice, one of the most serious diseases of rice worldwide (Nino-Liu et al. 2006). A range of virulence factors, including extracellular polysaccharide (EPS) synthesis, extracellular cellulase, and xylanase, and adhesions are produced by *X. oryzae* pv. *oryzae* to facilitate its pathogenesis (Das et al. 2009; White and Yang 2009). Like many other gram-negative bacteria, *X. oryzae* pv. *oryzae* also harbors a type III secretion system (T3SS) to inject

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and deliver effectors into plant cells, some of which are critical determinants of consequences during *X. oryzae* pv. *oryzae*–rice interactions. HrpG and HrpX are major regulatory proteins controlling T3SS expression (Song and Yang 2010; White and Yang 2009) in *X. oryzae* pv. *oryzae*. In the genome of *X. oryzae* pv. *oryzae* $PXO99^A$, 26 genes encoding GGDEF, EAL, or HD-GYP domain proteins were identified through bioinformatics analysis. Previous studies have demonstrated that some of these proteins were involved in the regulation of EPS production and virulence in *X. oryzae* pv. *oryzae*. PdeR, a response regulator with both GGDEF and EAL domains, was a PDE for c-di-GMP degradation, and PdeK/PdeR constituted a two-component system to regulate virulence in *X. oryzae* pv. *oryzae* (Yang et al. 2012). The HD-GYP domain protein RpfG is also involved in the regulation of virulence through the histidine kinase RpfC, which senses the diffusible signal factor (Andrade, et al. 2006; He et al. 2010). It is suggested that there might be a complicated c-di-GMP signaling network in *X. oryzae* pv. *oryzae* (Salzberg et al. 2008).

In this work, we characterized a putative signal protein (PXO_00403) which contains degenerate GGDEF and EAL domains similar to FimX, Thus, it was named the FimX-like protein (Filp). Our evidence showed that Filp bound to c-di-GMP through the EAL domain in vitro. Most importantly, *filp* was required for the full virulence of *X. oryzae* pv. *oryzae* on rice and induction of a hypersensitive response (HR) on non-host tobacco plants. Specific interactions between Filp and PliZ-domain protein PXO_02715 seems to be important for the function of Filp. These results imply that Filp acts as a novel c-di-GMP receptor to regulate the virulence expression in *X. oryzae* pv. *oryzae*.

RESULTS

The GGDEF and EAL domains of Filp are degenerate and enzymatically inactive.

Structural analysis and sequence alignments have identified some highly conserved residues that are crucial for the catalytic activity of GGDEF and EAL domains (Chan et al. 2004; De et al. 2008; Minasov et al. 2009; Navarro et al. 2009; Rao et al. 2008; Tchigvintsev et al. 2010; Wassmann et al. 2007). Filp is a putative signal protein containing both GGDEF and EAL domains (Fig. 1A). Alignment analysis of GGDEF and EAL domains of Filp with those experimentally confirmed active enzymes revealed that Filp lost most of the conserved residues (Fig. 1). For example, the GGDEF motif is changed to GEHSF in Filp. Two residues (K^{442} and R^{446}) of PleD, which mediate GTP binding, are substituted by A^{419} and G^{423} in Filp. The inhibitory site (I-site), which has the conserved motif RxxD, was replaced by DxxV in Filp (Fig. 1B). In the EAL domain, the signature motif is changed to QAF. Three residues (N^{237}, E^{269}, and D^{300}) of RocR, which are required for Mg^{2+} binding, are replaced by R^{534}, Q^{566}, and Q^{597} in Filp (Fig. 1B).

To experimentally test the DGC and PDE activities of Filp, we cloned the full length of *filp* gene into expression vector pColdSUMO, which generates an N-terminal SUMO-His_6 tag to increase the solubility of target proteins. Recombinant protein SUMO-His_6-Filp was purified. The DGC or PDE activity was tested by incubation of the purified Filp protein with the substrate GTP or c-di-GMP. High-pressure liquid chromatography (HPLC) was performed to analyze the products from the reaction. No synthesis or degradation of c-di-GMP was detected in the specified conditions (Supplementary Fig. S1), suggesting that Filp is DGE and PDE inactive. This result is consistent with the in silico analysis mentioned above. PdeRGE, which was confirmed to be a PDE, was used as a positive control for this experiment.

Filp binds to c-di-GMP with high affinity in vitro.

Proteins containing degenerate GGDEF and EAL domains have been shown to perform biological functions by acting as c-di-GMP receptors (Schirmer and Jenal 2009). Both in silico analysis and enzyme assays revealed that GGDEF and EAL domains of Filp were degenerate, and alignment of Filp with the inactive control FimX and LapD indicated that it might be a FimX-like protein (Fig. 1B). To determine whether Filp functions as a c-di-GMP receptor, we used isothermal titration calorimetry (ITC) to detect the binding affinity between Filp and c-di-GMP. In this assay, the purified recombinant protein SUMO-His_6-Filp or SUMO-His_6 was titrated with c-di-GMP at 20°C. The heat released during the process was recorded and the data were analyzed by the Origin software. The dissociation constant (*Kd*) was calculated from the association constants derived from the ITC results. Filp binds c-di-GMP in a 1:1 stoichiometric ratio, with an estimated *Kd* of 198 ± 9 nM (Fig. 2A). As expected, no physical interaction was observed between the control protein SUMO-His_6 and c-di-GMP (Fig. 2B). These findings indicated that Filp bound c-di-GMP with high affinity.

The EAL domain of Filp is critical for c-di-GMP binding.

BlastP searches revealed that Filp contained REC (12 to 121 amino acids [aa]), PAS (143 to 232 aa), GGDEF (270 to 426 aa), and EAL (447 to 683 aa) domains (Fig. 1A). To determine which domain of Filp participates in binding with c-di-GMP, Filp proteins lacking the REC (FilpΔR), PAS (FilpΔP), GGDEF (FilpΔG), or EAL (FilpΔE) domain were expressed, and the purified proteins were assayed via ITC for c-di-GMP binding. The results indicated that the truncated protein FilpΔR, FilpΔP, or FilpΔG still bound c-di-GMP with 1:1 stoichiometry (Fig. 2C through E). In contrast, the truncated protein FilpΔE lost the binding ability (Fig. 2F), suggesting that the EAL domain might contain critical sites for c-di-GMP binding. To further define the critical sites within the EAL domain, point mutations were generated in the residues of Q^{477}, F^{479}, R^{481}, S^{490}, D^{508}, E^{596}, K^{617}, E^{653}, and F^{654} in Filp corresponding to the residues Q^{40}, F^{42}, R^{44}, S^{53}, D^{71}, E^{159}, K^{180}, E^{216}, and F^{217}, respectively, in *Xcc*$FimX^{EAL}$ (Fig. 1C), which were shown to bind c-di-GMP in the crystal structure of the *Xcc*$FimX^{EAL}$–c-di-GMP complex (Chin et al. 2012). Similar ITC experiments were performed on these protein mutants. Mutations of $F^{479}A$, $R^{481}A$, $D^{508}A$, $S^{490}A$, $K^{617}A$, and $E^{596}A$ still bind with c-di-GMP but Flip carrying mutations of $Q^{477}A$, $E^{653}A$, and $F^{654}A$ lost the ability of binding to c-di-GMP (Fig. 1C; Supplementary Table S2). These data suggested that the EAL domain and residues Q^{477}, E^{653}, and F^{654} were indispensable for Filp to bind c-di-GMP.

Filp is required for the full virulence of *X. oryzae* pv. *oryzae*.

Studies in pathogenic bacteria have revealed that the c-di-GMP signaling pathway plays a key role in regulation of virulence (Kozlova et al. 2011; Pitzer et al. 2011; Yang et al. 2012). To characterize which phenotypes are mediated by Filp, we constructed a *filp* gene deletion mutant in *X. oryzae* pv. *oryzae* $PXO99^A$. The effect of the *filp* mutation on *X. oryzae* pv. *oryzae* virulence was evaluated by performing pathogenicity assays on susceptible rice (*Oryza sativa* subsp. *japonica*). The bacterial cells of wild-type $PXO99^A$ and Δ*filp* were inoculated onto the tip of leaves as described below. The disease symptoms of rice were scored 14 days after bacterial inoculation. Compared with the wild-type strain, Δ*filp* caused less severe disease symptoms (Fig. 3A) and shorter lesion lengths (Fig. 3B), suggesting that its ability to infect the host plant was significantly impaired. When a plasmid was used to express the full length of *filp* in Δ*filp*, the diseased phenotypes were re-

stored (Fig. 3A and B). These results demonstrated that Filp was required for the full virulence of PXO99[A].

EAL, PAS, and REC domains are required for Filp to regulate *X. oryzae* pv. *oryzae* virulence.

To evaluate the importance of each domain of Filp in vivo, we also used truncated proteins FilpΔR, FilpΔP, FilpΔG, and FilpΔE for the complementation of Δ*filp*. Pathogenicity assays showed that expression of Filp and FilpΔG protein in Δ*filp* led to restoration of virulence to wild-type levels (Fig. 3A and B). In contrast, expression of FilpΔR, or FilpΔP, or FilpΔE protein in the Δ*filp* mutant failed to complement its virulence deficiency phenotypes. Western blotting analysis with His_6 tag showed that proteins are all expressed appropriately in the Δ*filp* mutant (data not shown). These results indicated that the EAL, PAS, and REC domains of Filp were required for its proper function in vivo.

Filp positively regulates T3SS expression.

c-di-GMP signaling interfering with T3SS expression has been reported previously in *P. aeruginosa* and *Dickeya dadantii* (Kulasakara et al. 2006; Moscoso et al. 2011; Yi et al. 2010). Based on the observation of changes in virulence of Δ*filp*, we speculated that the function of its T3SS might be affected. To determine whether deletion of *filp* has an effect on T3SS expression, we carried out quantitative reverse-transcription polymerase chain reaction (qRT-PCR) experiments to measure the transcriptional levels of T3SS regulator genes *hrpG* and *hrpX* and harpin gene *hpa1*. Expression of the *hrp* genes were induced in XOM2 minimal media (Furutanin et al. 2003) be-

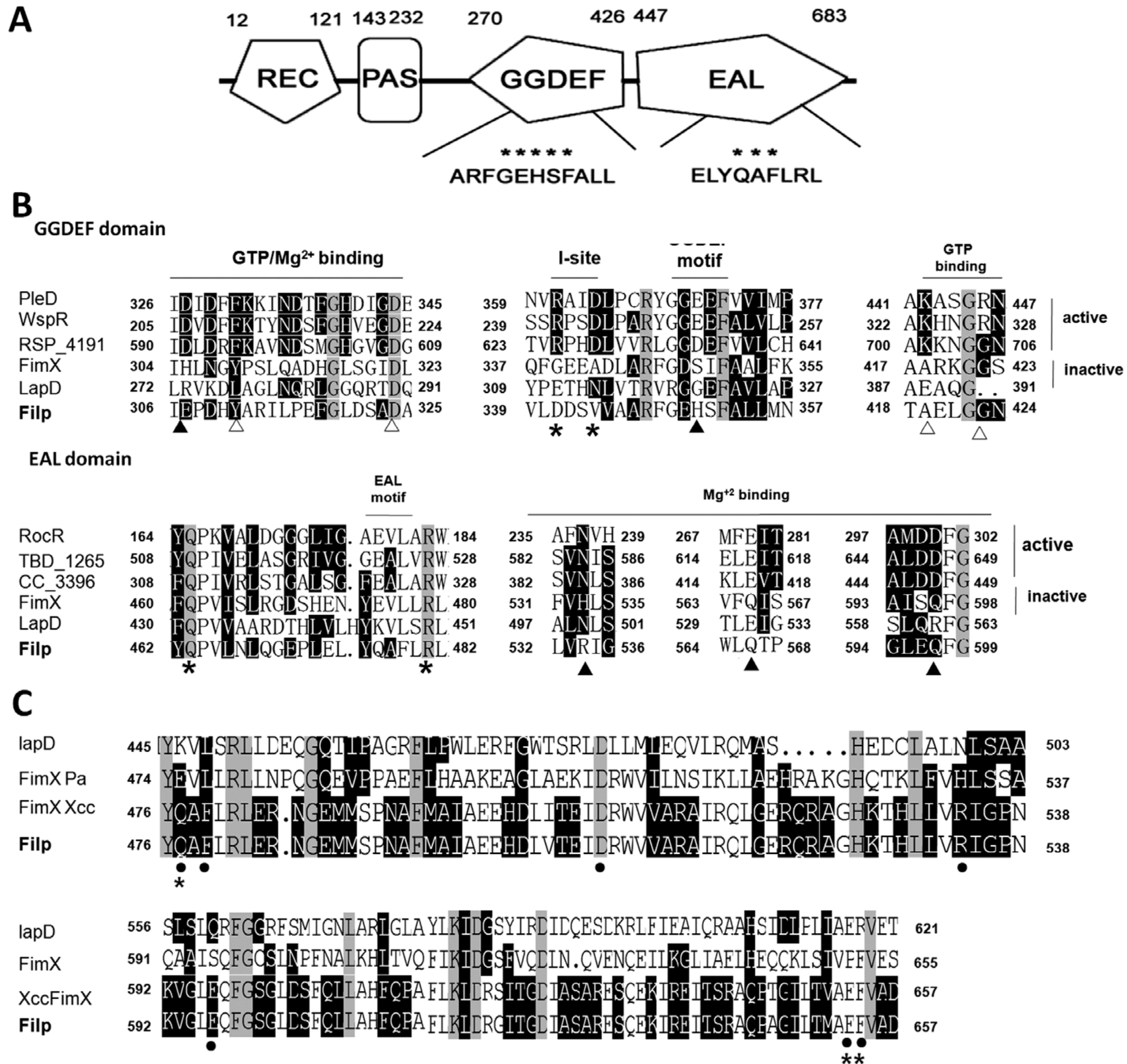

Fig. 1. GGDEF and EAL domains of Filp are degenerate. **A,** Schematic representation of the domain organization of Filp from *X*anthomonas *oryzae* pv. *oryzae* strain PXO99[A]. Numbers represent amino acid residues where the predicted domains start and end, based on the National Center for Biotechnology Information's conserved domain database and SMART database. REC = REC domain; PAS = PAS domain; GGDEF = GGDEF domain; EAL = EAL domain. Star indicates the conserved residues in the GGDEF and EAL domains of Filp. **B,** Sequence alignment of the GGDEF domain of Filp with those of PleD, WspR, and RSP_4149 representing enzymatically active domains, and FimX and LapD representing enzymatically inactive domains. Residues involved in magnesium binding (filled triangles), GTP binding (open triangles), and c-di-GMP binding (asterisks) were highlighted. Sequence alignment of the EAL domain of Filp with those of RocR, TBD1265, and CC3396 representing enzymatically active domains, and FimX and LapD representing enzymatically inactive domains. Residues involved in catalytic activity (asterisks) and magnesium binding (filled triangles) were highlighted. **C,** Sequence alignment of the EAL domain of Filp with those of LapD and FimX-like proteins representing c-di-GMP binding domains. Putative binding site residues (black dots) and residues confirmed by isothermal titration calorimetry assays (asterisks) involved in c-di-GMP binding were highlighted.

fore total RNA was extracted from the wild-type PXO99[A] and Δ*filp* strains. cDNAs were obtained and analyzed by qRT-PCR, as described below. The expressions of *hrpG*, *hrpX*, and *hpa1* were inducible in wild-type PXO99[A] grown in XOM2 but not in M210 (data not shown). The transcript level of each gene in Δ*filp* relative to that in the wild type, which was designated as 1, was calculated. The mRNA levels of *hrpX*, *hrpG*, and *hpa1* in Δ*filp* dropped to approximately 80, 50, and 40% of the wild-type level, respectively (Fig. 3C), and the differences were statistically significant ($P < 0.05$), suggesting that the mutation of the *filp* gene has a negative effect on *hrp* gene expression. Complementation of Δ*filp* by in trans expression of *filp* restored the gene expression to near wild-type level. These observations demonstrated that Filp positively regulated T3SS expression at the transcriptional level.

Δ*filp* is not defective in EPS production, biofilm formation, and flagellar motility.

Because Δ*filp* displayed attenuated virulence on rice, we investigated whether it is due to deficiency of EPS production, which has been regarded as an important virulence factor of *X. oryzae* pv. *oryzae*. However, no significant difference was found between Δ*filp* and PXO99[A] in term of EPS secretion by examining the colony and quantifying EPS production (Supplementary Fig. S2A and B), indicating that Filp does not regulate the EPS secretion of PXO99[A]. Moreover, c-di-GMP in many bacteria has been shown to regulate lifestyle transitions (i.e., a higher concentration of c-di-GMP facilitates biofilm formation while lower concentrations induce flagellar motility) (Schirmer and Jenal 2009). Therefore, we tested whether a mutation of *filp* affected biofilm formation and flagellum-dependent swim-

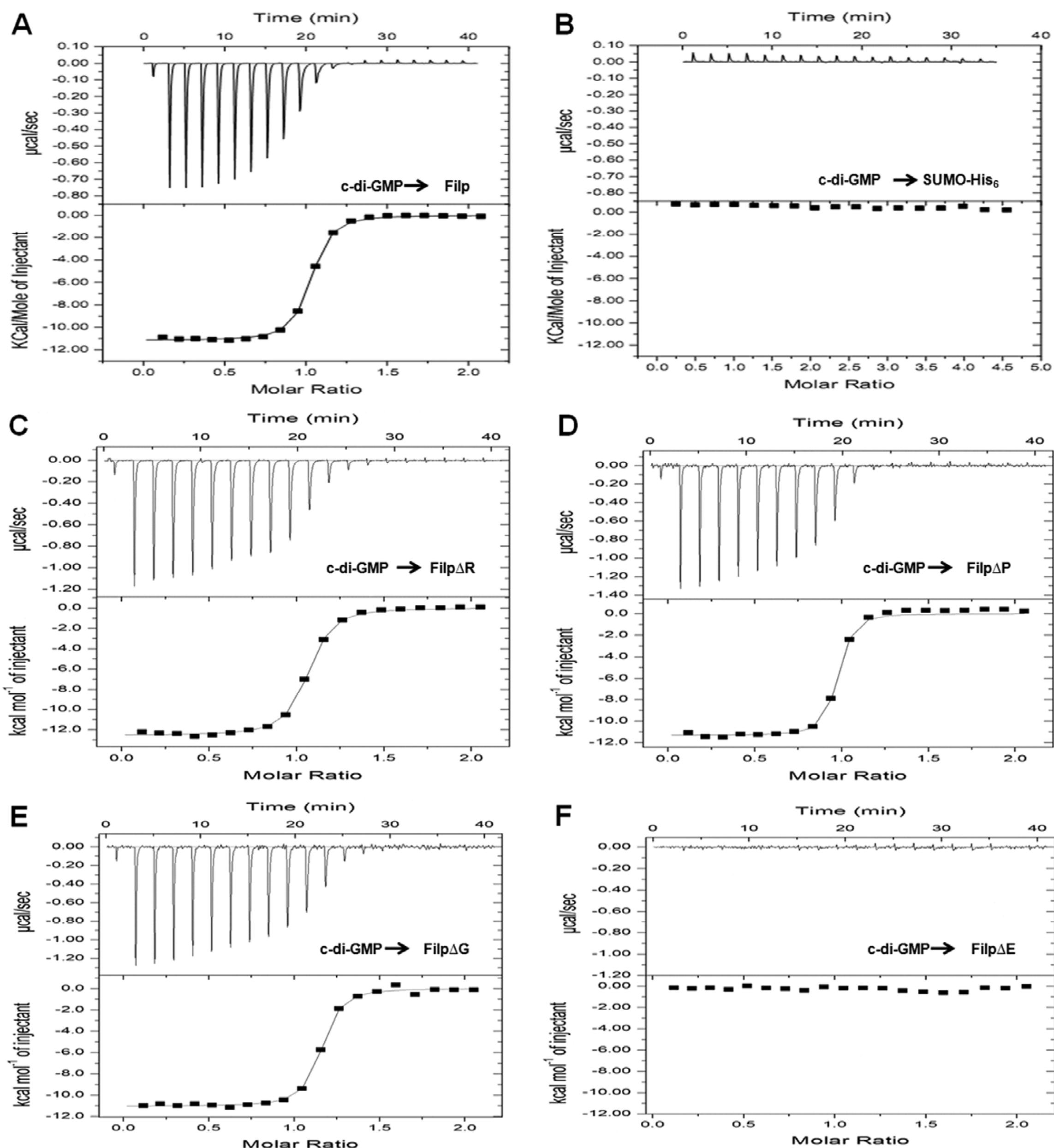

Fig. 2. Isothermal titration calorimetry (ITC) analysis of c-di-GMP binding to Filp. **A,** Binding of c-di-GMP to Filp, which was purified as the recombinant protein SUMO-His_6-Filp. Top panel shows the titration calorimetry of 20 μM Filp with 2-μl aliquots of 500 μM c-di-GMP at 20°C. Normalized ITC data for titrations versus molar ratio of Filp and c-di-GMP are shown on the lower panels. Data analysis using Origin 7.0 indicates that the binding data fit well to a single-site binding model. **B,** Binding of c-di-GMP to the control protein SUMO-His_6. **C,** Binding of c-di-GMP to the truncated protein FilpΔR. **D,** Binding of c-di-GMP to the truncated protein FilpΔP. **E,** Binding of c-di-GMP to the truncated protein FilpΔG. **F,** Binding of c-di-GMP to the truncated protein FilpΔE.

ming motility in PXO99A. No significant change in biofilm formation between Δ*filp* and PXO99A was observed. Strain Δ*filp* showed swimming sizes similar to that of the wild type on semisolid media plates containing 0.3% agar, whereas Δ*fliC* as a control showed significantly impaired swimming motility. These observations suggested that Filp did not regulate EPS production, biofilm formation, and flagellar motility. In addition, we tested the sliding motility of Δ*filp* and Δ*fliC* on SB (described below) media plates containing 0.6% agar, which have been used for analyzing the sliding motility of other *Xanthomonas* strains. Strain Δ*filp* but not Δ*fliC* showed increased sliding zones, indicating that Filp regulated sliding motility similar to FimX-like proteins in *X. campestris* pv. *campestris* and *X. axonopodis* pv. *citri*.

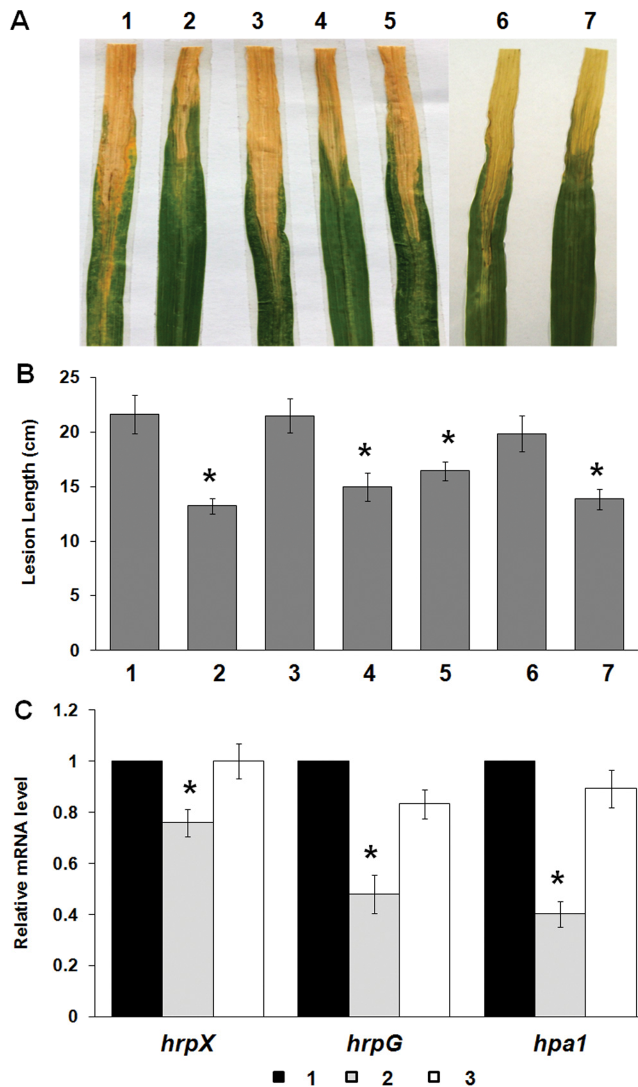

Fig. 3. The Δ*filp* mutant showed attenuated virulence on rice and type III secretion system gene expression. **A,** *Xanthomonas oryzae* pv. *oryzae* strains were inoculated on rice using the leaf clipping method. Bacterial blight symptom was observed 14 days after inoculation. **B,** Diseased lesion lengths were recorded. The error bar represents standard deviation of the lesion lengths from at least 10 leaves. **C,** Relative transcript levels of *hrpG*, *hrpX*, and *hpa1* in Δ*filp* were determined by quantitative reverse-transcription polymerase chain reaction analysis using the $2^{-\Delta\Delta CT}$ method. The housekeeping gene *gyrB* was used as a reference gene. The expression level of each gene in the wild type was mathematically designated as 1 for each reaction. The experiment was repeated three times with similar results. Columns: 1 = wild-type PXO99A, 2 = Δ*filp*, 3 = Δ*filp*(pBFilp), 4 = Δ*filp*(pBFilpΔR), 5 = Δ*filp*(pBFilpΔP), 6 = Δ*filp*(pBFilpΔG), and 7 = Δ*filp*(pBFilpΔE); * indicates $P < 0.05$ by *t*-test.

Filp specifically interacts with the PilZ-domain protein PXO_02715.

c-di-GMP-dependent signaling pathways have been reported to regulate bacterial motility through protein–protein interactions (Pultz et al. 2012). FimX-like proteins have been shown to mediate motility mainly through interacting with a PilZ-domain protein (Qi et al. 2012). Bioinformatics analysis indicated that there are three PilZ-domain proteins (PXO_02715, PXO_02374, and PXO_00049) in the PXO99A genome. Thus, we surmised that Filp might also interact with them in *X. oryzae* pv. *oryzae*. We carried out yeast two-hybrid (Y2H) assays to test the interactions between them. We constructed plasmids pGBKFilp, pGAD2715, pGAD2374, and pGAD49 to express fusion proteins DBD-Filp, AD-PXO_2715, AD-PXO_2374, and AD-PXO_00049, respectively. The resulting plasmids were transformed into *Saccharomyces cerevisiae* strains, and protein expressions at expected sizes were detected by Western blotting analysis (Supplementary Fig. S3). Interactions between the fusion proteins were screened for growth on the quadruple drop-out (QDO) media lacking amino acids Trp, Leu, His, and Ade. The results showed that the yeast diploids containing DBD-Filp/AD-2715 grew well on QDO media, while those containing DBD-Filp/AD-PXO_02374, DBD-Filp/AD-PXO_00049, and the negative control DBDFilp-AD/DBD-PXO_02715AD did not grow (Fig. 4A). In order to double confirm the interactions, we employed glutathione-*S*-transferase (GST) pull-down experiments. GST-Filp, GST tag only, SUMO-His_6 tag only, and SUMO-His_6-PXO_02715 were expressed and purified (Supplementary Fig. S4). Different combinations of the purified proteins were mixed and then precipitated by the GST-binding beads. Then, the eluted proteins were analyzed by Western blotting using anti-His or anti-GST antibodies. SUMO-His_6-PXO_02715 protein was only detected when GST-Filp was present in the mixture, indicating Filp was able to pull down PXO_02715 (Fig. 4B). These results demonstrated that Filp specifically interacted with PXO_02715.

Δ*PXO_02715* displays impaired virulence and reduced T3SS expression similar to that of Δ*filp*.

Specific interaction between Filp and PXO_02715 suggests that Filp and PXO_02715 might be functionally related in *X. oryzae* pv. *oryzae*. To demonstrate whether PXO_02715 affects bacterial virulence expression in PXO99A, we constructed the deletion mutant Δ*PXO_02715* and the complemented strain. The virulence assay showed that deletion of *PXO_02715* led to reduced virulence on rice and caused a shorter disease lesion length (Fig. 5A and B). In trans expression of the *PXO_02715* gene in the Δ*PXO_02715* mutant background restored the pathogenic phenotype to the wild-type level. Moreover, we tested the T3SS gene expression in Δ*PXO_02715*. Results from the qRT-PCR experiments indicated that expression of *hrpX, hrpG,* and *hpa1* genes was downregulated to approximately 37, 39, and 50% of the wild-type level, respectively (Fig. 5C). These data implied that PXO_02715 might work together with Filp in the same pathway to regulate virulence expression of *X. oryzae* pv. *oryzae*.

Δ*filp* and Δ*PXO_02715* do not trigger HR on nonhost tobacco plants.

When infiltrated on nonhost plants, pathogenic bacteria often elicit the HR, a type of programmed cell death resulting from the plant defense mechanism (Dangl et al. 1996). Because Filp and PXO_02715 positively regulated T3SS gene expression, whose function is often required for triggering nonhost HR, we decided to test the phenotypes of Δ*filp* and Δ*PXO_02715* on tobacco plants (*Nicotiana benthamiana*). Different bacterial strains were infiltrated in tobacco leaves at the

concentration of 10^8 cells/ml. The results showed that wild-type PXO99^A and the complemented strains Δ*filp* (pBFilp) and Δ*PXO_02715* (pB2715) induced HR at 24 h post inoculation, whereas Δ*filp* and Δ*PXO_02715* did not (Fig. 6). These observations suggested that Filp and PXO_02715 were required for PXO99^A to induce HR on tobacco, which is consistent with their roles as positive regulators for T3SS gene expression.

DISCUSSION

The GGDEF and EAL domain-containing proteins can fall into three categories, based on whether they are enzymatically active. PdeA from *Caulobacter crescentus* and PdeB from *Shewanella oneidensis* were identified as the c-di-GMP-specific PDE (Chao et al. 2013; Christen et al. 2005). In *Vibrio parahaemolyticus,* ScrC exhibits both PDE and DGC activities in certain conditions (Ferreira et al. 2008). The degenerate GGDEF and EAL domain proteins represent a major type of c-di-GMP receptors or effectors, such as FimX and LapD (Schirmer and Jenal 2009; Sondermann et al. 2012). In this report, we identified and characterized Filp, a c-di-GMP-binding protein containing GGDEF and EAL domains from *X. oryzae* pv. *oryzae*. Both in silico analysis and enzyme assays demonstrated that GGDEF and EAL domains of Filp were degenerate and enzymatically inactive (Fig. 1). Most importantly, ITC data showed that Filp bound c-di-GMP with high affinity in a 1:1 stoichiometric ratio in the EAL domain, whereas the GGDEF domain was not needed for such binding (Fig. 2). These experimental results are accordant with the bioinformatic analysis of the domains of the Filp protein. The c-di-GMP-bound structure of FimX^{EAL} showed that residues Q^{477} and F^{654} in the EAL domain participate in the interaction with c-di-GMP (Chin et al. 2012). In addition to Q^{477} and F^{654} of Filp, which were corresponding to Q^{477} and F^{654} of FimX (Fig. 1C), we found that E^{653} of Filp was also required for the binding with c-di-GMP. Because these residues were in the EAL domain of Filp, we concluded that the. EAL domain of Filp was essential for c-di-GMP binding. More detailed information about binding function will be further required through solving the crystal structure of Filp in complex with c-di-GMP.

c-di-GMP receptors or effectors have been illustrated to be the most critical components to regulate the downstream phenotypes (i.e., virulence via the c-di-GMP signaling pathway). They sense and translate the fluctuation of the c-di-GMP concentration into cellular behaviors by various mechanisms (Sondermann et al. 2012). Several different types of c-di-GMP receptors have been identified in other *Xanthomonas* spp. For example, the PliZ domain, a widely conserved c-di-GMP-binding module, was recently experimentally confirmed in *X. campestris* pv. *campestris* (Li et al. 2011). Moreover, transcriptional regulator Clp from *X. campestris* pv. *campestris* and *X. axonopodis* pv. *citri,* which has cNMP- and DNA-binding domains, was shown to interact with c-di-GMP specifically (Leduc and Roberts 2009; Tao et al. 2010). In this study, we reported Filp as the first functional c-di-GMP receptor identified in *X. oryzae* pv. *oryzae*. Filp showed protein sequence similarities of 96.8 and 93.8% to $FimX_{XAC2398}$ and XccFimX, respectively, suggesting that the c-di-GMP signaling pathway mediated by the FimX-like proteins might be conserved in these pathogenic species. Although FimX-like proteins in *Xanthomonas* strains have been previously reported to regulate bacterial motility (Guzzo et al. 2009, 2013), the relationship between them and bacterial virulence has not yet been demonstrated. Here, we showed the first evidence that Filp specifically regulated the virulence of *X. oryzae* pv. *oryzae* on rice, whereas it did not regulate EPS production, biofilm formation, or swimming motility.

Based on the observations that the EAL domain of Filp was necessary for c-di-GMP binding in vitro and for full complementation of the virulence-deficient phenotype of Δ*filp* (Figs. 3 and 4), we concluded that the EAL domain was the most important for the function of Filp. In addition, the REC and PAS domains, which were not involved in c-di-GMP binding, were probably required for the proper function of Filp in vivo (Figs. 3 and 4). REC domain is the signal-sensing domain from the sensor partner in the two-component systems, and PAS domain normally senses environmental signals such as oxygen, redox, and light (Mao et al. 2012; Sousa et al. 2013). The existence and importance of the REC and PAS domains in Filp suggested that multiple environmental signal clues might also participate in this signal transduction pathway.

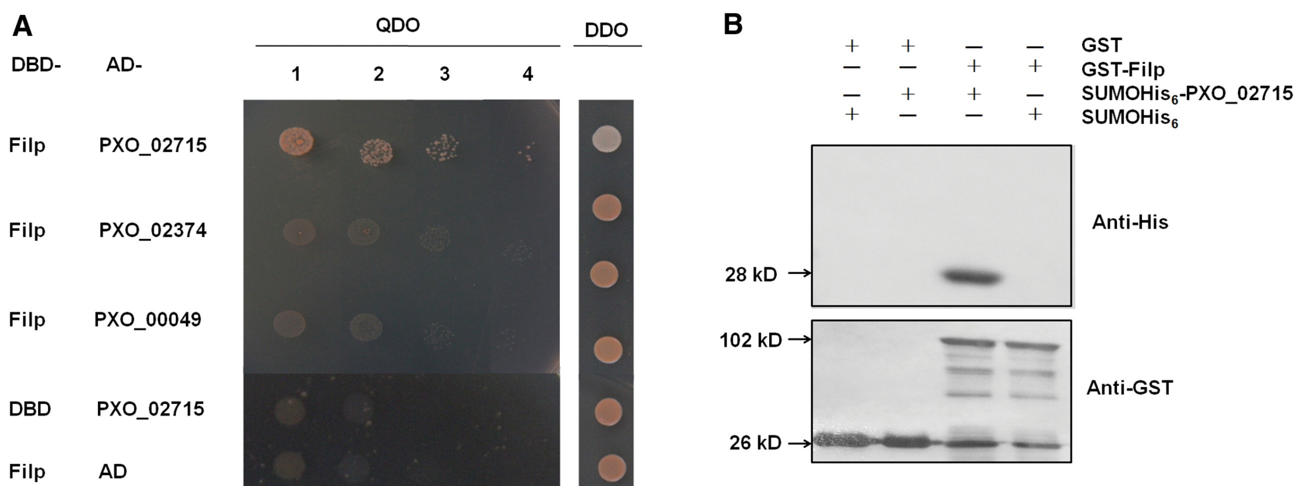

Fig. 4. Specific interaction between Filp and PilZ-domain protein PXO_02715 was detected. **A,** Yeast two-hybrid (Y2H) assays to show the interactions between Filp and the PilZ-domain proteins. Y2HGold yeast strains carrying pGBKFilp and Y187 yeast strains carrying pGAD2715, pGAD2374, and pGAD49 were mated and selected on double drop-out (DDO) plates. Diploids capable of growth on DDO were further examined on quadruple drop-out (QDO) plates for the interactions between test proteins. Serial dilutions (10-fold) were performed to evaluate the strength of the interactions observed. Pictures were taken after the cells grew on DDO or QDO plates for 48 h. **B,** Glutathione-*S*-transferase (GST) pull-down analysis of interaction between Filp and PXO_02715. Purified GST-Filp fusion protein or GST was incubated with equal amount of purified SUMO-His_6-PXO_02715 or SUMO-His_6, and accreted to glutathione-sepharose beads. Beads were then washed and eluted proteins were analyzed by Western blots using anti-His (upper) or anti-GST (lower) antibodies. Protein sizes were labeled on the left of membrane.

It has been recognized that interactions of c-di-GMP receptors with protein partners are often involved in the downstream signaling mechanisms (Paul et al. 2010; Steiner et al. 2013). In *Xanthomonas* spp., PilZ-domain proteins have been shown to regulate motility (McCarthy et al. 2008; Ryan et al. 2012). Recently, it has been reported that FimX, PilZ protein XAC1133, and PilB ATPase formed a complex to promote the T4P formation and inhibit the sliding motility (Guzzo et al. 2009, 2013; Qi et al. 2012). Here, we identified PXO_02715 as a Filp-interacting protein with a PilZ domain from *X. oryzae* pv. *oryzae*. The PilZ domain of PXO_02715 was degenerate and lost the binding activity with c-di-GMP (*unpublished data*). Genetic analysis revealed that PXO_02715 was also a positive regulator of bacterial virulence on rice and HR induction on tobacco, which was similar to Filp (Figs. 5 and 6). We speculated that Filp and PXO_02715 might function together in the same pathway.

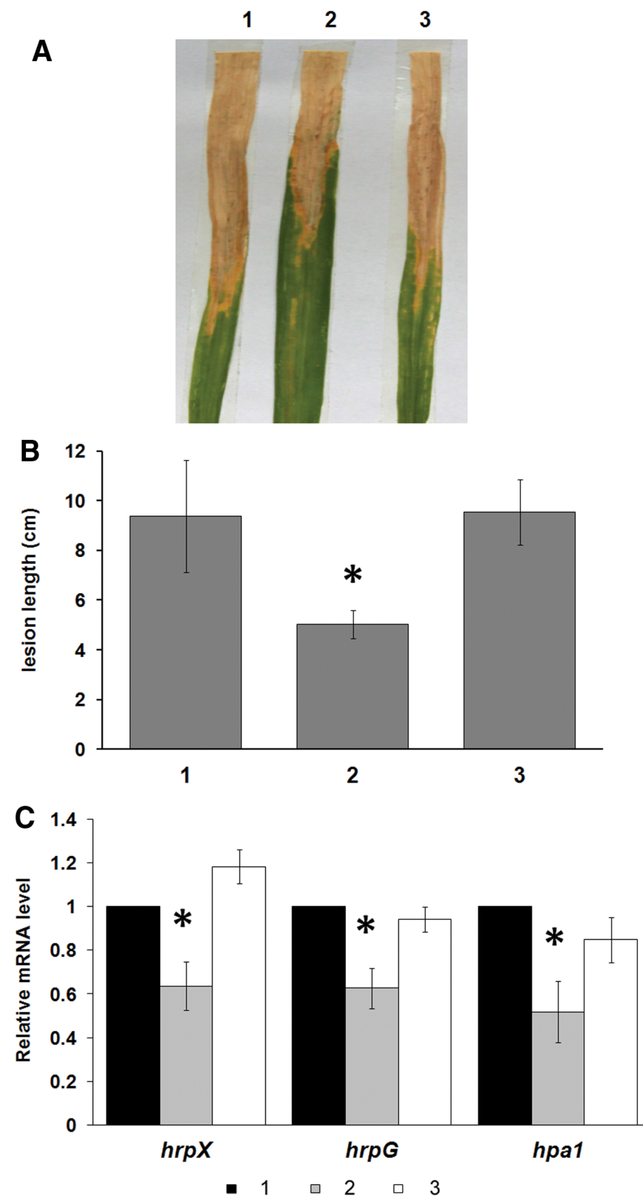

Fig. 5. The Δ*PXO_02715* mutant showed impaired virulence on rice and reduced type III secretion system gene expression. **A,** Bacterial blight symptom was observed 14 days after *Xanthomonas oryzae* pv. *oryzae* strain inoculation on rice. **B,** Diseased lesion lengths were recorded 14 days after inoculation. The error bar represents the standard deviation of three independent replicates. **C,** Relative transcript levels of *hrpG*, *hrpX*, and *hpa1* in Δ*PXO_02715* were determined by quantitative reverse-transcription polymerase chain reaction. Columns: 1 = wild-type PXO99^A, 2 = Δ*PXO_02715*, and 3 = Δ*PXO_02715*(pB2715); * indicates $P < 0.05$ by *t*-test.

Previous work by others on the metabolism of c-di-GMP showed that the intracellular level of c-di-GMP has an effect on the expression of the T3SS in plant-pathogenic bacteria (Moscoso et al. 2011; Yang et al. 2012; Yi et al. 2010; Zhang et al. 2013) but the relationship between c-di-GMP receptors and T3SS expression was unknown. In *X. campestris* pv. *campestris*, the PilZ-domain proteins XC0965 and XC2317, which were identified as c-di-GMP receptors, were regulated by HrpG and HrpX (McCarthy et al. 2008). This raises the possibility that c-di-GMP receptors might be involved in T3SS regulation. We showed that Filp and its partner protein PXO_02715 regulated *hrp* gene expression (Figs. 3 and 5), which probably explains why Δ*filp* and Δ*PXO_02715* were attenuated in bacterial virulence in susceptible rice (Figs. 3 and 5). This is also consistent with the observation that Filp and PXO_02175 were required for *X. oryzae* pv. *oryzae* to trigger HR on tobacco (Fig. 6). The intracellular c-di-GMP level in *X. oryzae* pv. *oryzae* were regulated by DGC and PDE, such as a phosphodiesterase PdeR, whose activity can be affected by the phosphorylation process in a two-component system (Yang et al. 2012), or various environmental signals, which are unknown to date. Subsequently, c-di-GMP sensed by the receptor protein Filp regulates virulence through T3SS expression and this regulation might be through Filp's interacting-partner protein PXO_02715. Although we do not know how they work together to regulate T3SS expression, it is plausible for us to propose that the concentration of intracellular c-di-GMP might influence the interaction between Filp and PXO_02715 and, thus, interfere with their downstream signaling mechanism. Therefore, further elucidation of the effect of intracellular c-di-GMP level on the function of Filp and PXO_02715 will help us to reveal the c-di-GMP signaling mechanism in *X. oryzae* pv. *oryzae*.

MATERIALS AND METHODS

Bacterial strains and growth conditions.

The bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* strains were grown in Luria-Bertani medium at 37°C (Hanahan 1983). *X. oryzae* pv. *oryzae* PXO99^A (Hopkins et al. 1992) and derived mutants strains

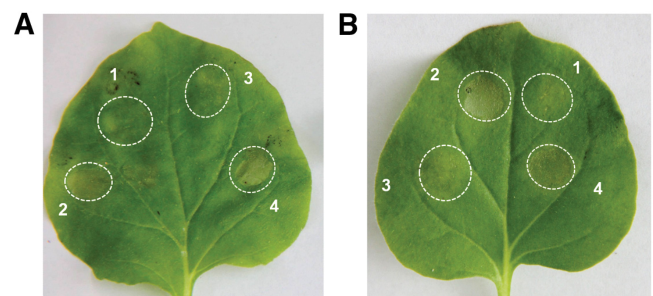

Fig. 6. Both Δ*filp* and Δ*PXO_02715* lost the ability of hypersensitive response (HR) induction in nonhost tobacco leaves. Bacterial cell suspensions at the concentration of 10^8 cells/ml were infiltrated into tobacco leaves. Photos were taken at 24 h post infiltration. The infiltrated region was indicated by white dotted line. At least 10 leaves were inoculated for each strain per experiment. The experiment was repeated five times. **A,** Δ*filp* did not trigger HR: circle 1 = 10 mM sodium phosphate buffer, circle 2 = wild-type PXO99^A, circle 3 = Δ*filp*, and circle 4 = Δ*filp*(pBFilp). **B,** Δ*PXO_02715* did not induce HR: circle 1 = 10 mM sodium phosphate buffer, circle 2 = wild-type PXO99^A, circle 3 = Δ*PXO_02715*, and circle 4 = Δ*PXO_02715* (pB2715).

were cultured at 28°C on peptone sucrose agar (PSA) medium or M210 liquid medium with appropriate antibiotics. XOM2 medium (Furutanin et al. 2003) was used to induce the expression of T3SS genes. The antibiotics used were ampicillin, kanamycin (Km), and gentamycin (Gm) at concentrations of 100, 50, and 20 μg ml^{-1}, respectively.

Protein expression and purification.

The DNA fragments encoding Filp, FilpΔR, and FilpΔE of Filp were amplified by primer pairs FilpPF1/FilpPR1, FilpPF2/FilpPR1, and FilpPF1/FilpPR2, respectively. To obtain the fragment *filpΔP*, we first amplified the REC domain and GGDEF-EAL domain separately with primers FilpPF1/FilpPR3 and FilpPF3/FilpPR1. Then, FilpPF1/FilpPR1 was used to amplify *filpΔP*, using the two fragments as template. We also amplified the REC-PAS domain and EAL domain separately with primers FilpPF1/FilpPR4 and FilpPF4/FilpPR1; then, FilpPF1/FilpPR1 was used to amplify *filpΔG* using two fragments as the templates. The DNA fragment encoding PXO_02715 were amplified by primer pair 2715PF/2715PR. PCR fragments were gel purified and cloned to the middle vector pMD18-T, following verification by sequencing. Finally, the full length of *filp* was cloned into expression vector pColdSUMO or pGEX-6P-1, resulting in pCFilp or pGFilp. The other truncated fragments of *filp* and full-length *PXO_02715* were cloned into pColdSUMO, resulting in plasmids pCFilpΔR, pCFilpΔP, pCFilpΔG, pCFilpΔE, and pC2715. The plasmids were then transformed into *E. coli* BL21 strains for protein expression.

For purification of the SUMO-His_6 fusion proteins, the following protocol was used. Briefly, expression of the target protein was induced by addition of isopropyl-thio-galactopyranoside at a final concentration of 0.3 mM. The bacterial cultures were then incubated at 16°C for 8 h; then, the cells were chilled to 4°C and collected by centrifugation. The cell pellets were resuspended in phosphate-buffered saline (PBS), followed by sonication. The crude cell extracts were centrifuged at 12,000 × *g* for 20 min. The soluble protein fractions were collected and mixed with pre-equilibrated Ni_2 resin (GE Healthcare, Piscataway, NJ, U.S.A.) for 2 to 3 h at 4°C, then placed into a column and extensively washed with buffer con-

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics[a]	Reference or source
Escherichia coli		
DH5α	supE44 Δ*lacU169*(Φ*80lacZΔM15*) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Hanahan 1983
BL21	For protein expression	Novagen
Xanthomonas oryzae pv. *oryzae*		
$PXO99^A$	Wild-type strain, Philippine race 6	Lab collection
Δfilp	*filp* gene deletion mutant derived from $PXO99^A$, Gm^r	This study
ΔPXO_02715	*PXO_02715* gene deletion mutant derived from $PXO99^A$, Gm^r	This study
Saccharomyces cerevisiae		
Y2HGold	*MATα, trp1-901, leu2-3, 112, ura3-52, his3-200, Δgal4, Δgal80, LYS2::GAL1*$_{UAS}$*-Gal1*$_{TATA}$*-His3, GAL2*$_{UAS}$*-Gal2*$_{TATA}$*-Ade2, URA3::MEL1*$_{UAS}$*-Mel1*$_{TATA}$*, AUR1-C MEL1*	Clontech
Y187	*MATα, ura3-52, his3-200, ade2-101, trp1-901, leu2-3, 112, Δgal4, Δgal80, met– URA3::GAL1UAS-GAL1TATA-lacZ, MEL1*	Clontech
Plasmid		
pColdSUMO	Expression vector to generate a N-terminal SUMO-His_6 tag, Ap^r	Haigene
pGEX-6P-1	Expression vector to generate a N-terminal GST tag, Ap^r	GE Healthcare
pK18mobsacB	Suicidal vector carrying *sacB* gene for mutagenesis, Km^r	Schafer et al. 1994
pBBR1MCS-4	Broad-host range expression vector, Ap^r	Kovach et al. 1995
pGADT7	Yeast-two hybrid vector to create fusion protein containing the GAL4 activation domain, Ap^r	Clontech
pGBKT7	Yeast two-hybrid vector to create fusion protein containing GAL4 DNA binding domain, Km^r	Clontech
pMD18-T	Cloning vector, Ap^r	Takara
pCFilp	pColdSUMO carrying the coding sequence for Filp, Ap^r	This study
pGFilp	pGEX-6P-1 carrying the coding sequence for Filp, Ap^r	This study
pC2715	pColdSUMO carrying the coding sequence for PXO_02715, Ap^r	This study
pCFilpΔR	pColdSUMO carrying the coding sequence for Filp mutant missing the REC domain, Ap^r	This study
pCFilpΔP	pColdSUMO carrying the coding sequence for Filp mutant missing the PAS domain, Ap^r	This study
pCFilpΔE	pColdSUMO carrying the coding sequence for Filp mutant missing the EAL domain , Ap^r	This study
pCFilpΔG	pColdSUMO carrying the coding sequence for Filp mutant missing the GGDEF domain, Ap^r	This study
pCFilpQ^{477}A	pColdSUMO carrying the coding sequence for the point mutation of Q^{477}A, Ap^r	This study
pCFilpF^{479}A	pColdSUMO carrying the coding sequence for the point mutation of Q^{479}A, Ap^r	This study
pCFilpR^{481}A	pColdSUMO carrying the coding sequence for the point mutation of R^{481}A, Ap^r	This study
pCFilpS^{490}A	pColdSUMO carrying the coding sequence for the point mutation of S^{490}A, Ap^r	This study
pCFilpD^{508}A	pColdSUMO carrying the coding sequence for the point mutation of D^{508}A, Ap^r	This study
pCFilpR^{534}A	pColdSUMO carrying the coding sequence for the point mutation of R^{534}A, Ap^r	This study
pCFilpE^{596}A	pColdSUMO carrying the coding sequence for the point mutation of E^{596}A, Ap^r	This study
pCFilpE^{653}A	pColdSUMO carrying the coding sequence for the point mutation of E^{653}A, Ap^r	This study
pCFilpF^{654}A	pColdSUMO carrying the coding sequence for the point mutation of F^{654}A, Ap^r	This study
pKFilpG	pK18mobSacB derivative with *filp* inserted by Gm resistance gene, Km^r, Gm^r	This study
pK2715G	pK18mobSacB derivative with *PXO_02715* inserted by Gm resistance gene, Km^r, Gm^r	This study
pBFilp	pBBR1MCS-4 carrying the full-length of *filp*, Ap^r	This study
pBFilpΔR	pBBR1MCS-4 carrying the coding sequence for Filp mutant missing the REC domain, Ap^r	This study
pBFilpΔP	pBBR1MCS-4 carrying the coding sequence for Filp mutant missing the PAS domain of filp, Ap^r	This study
pBFilpΔG	pBBR1MCS-4 carrying the coding sequence for Filp mutant missing the GGDEF domain, Ap^r	This study
pBFilpΔE	pBBR1MCS-4 carrying the coding sequence for Filp mutant missing the EAL domain, Ap^r	This study
pB2715	pBBR1MCS-4 carrying the full-length of *PXO_02715*, Ap^r	This study
pGBKFilp	pGBKT7 carrying the full length of *filp*, Km^r	This study
pGAD2715	pGADT7 carrying the full length of *PXO_02715*, Ap^r	This study
pGAD2374	pGADT7 carrying the full length of *PXO_02374*, Ap^r	This study
pGAD49	pGADT7 carrying the full length of *PXO_00049*, Ap^r	This study

[a] Ap^r, Km^r, and Gm^r indicate resistant to ampicillin, kanamycin, and gentamicin, respectively.

taining 20 mM Tris-HCl (pH 8.0), 350 mM NaCl, 0.5 mM EDTA, 10% glycerol, 5 mM $MgCl_2$, and 30 mM imidazole. The proteins were subsequently eluted with buffer containing 300 mM imidazole. The GST-fusion protein GST-Filp was purified through a GST-binding column (GE Healthcare). The soluble protein fractions were mixed with pre-equilibrated GST for 2 to 3 h at 4°C and washed with PBS buffer. The proteins were subsequently eluted with washing buffer containing 10 mM glutathione and 50 mM Tris-HCl, pH8.0. The fusion proteins were collected and analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The SUMO-His_6 tag expressed from the empty vector pColdSUMO and GST tag expressed from pGEX-6P-1 were purified using the same procedure as negative controls.

Construction of protein variants carrying mutations.

Point mutations of the conserved binding sites of Filp with c-di-GMP were generated by Bridge PCR. For the mutation of Q^{477}, the upstream and downstream fragments were amplified using the primers FilpPF1/Q^{477}R and Q^{477}F/FilpPR1 and the products were gel purified; then, using two fragments as the templates to amplify the fragment FilpQ^{477}A using the primer FilpPF1/FilpPR1, cloned to the pMD18-T and sequenced. pMDFilpQ^{477}A was treated with *Bam*HI/*Hin*dIII, then inserted to the pColdSUMO, resulting in pCFilpQ^{477}A. The point mutations for F^{479}, R^{481}, S^{490}, D^{508}, R^{534}, E^{596}, E^{653}, and F^{654} were constructed using the methods as pCFilpQ^{477}, resulting in pCFilpF^{479}A, pCFilpR^{481}A, pCFilpS^{490}A, pCFilpD^{508}A, pCFilpR^{534}A, pCFilpE^{596}A, pCFilpE^{653}A, and pCFilpF^{654}A, respectively. The plasmids were transferred into *E. coli* BL21 strains for protein expression. Detailed information on the primers is listed in Supplementary Table S1.

Enzyme assay and HPLC analysis.

HPLC was used to detect the production or degradation of c-di-GMP, as described previously (Ryjenkov et al. 2005). The DGC reaction system contains approximately 25 μg of purified proteins and 100 μM GTP in 1 ml of running buffer (75 mM Tris-HCl [pH 7.8], 250 mM NaCl, 25 mM KCl, and 10 mM $MgCl_2$). The mixture was incubated at 37°C for 2 h. Sample (100 μl) was removed at regular intervals and the reaction stopped with 250 mM EDTA. The putative PDE activity of Filp was tested using the reaction system containing 20 μg of protein, 10 μM c-di-GMP, 50 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 10 mM $MnCl_2$, 0.5 mM EDTA, and 50 mM NaCl in a total volume of 1 ml. The mixture was incubated at 37°C for 2 h. Aliquots (each 100 μl) were withdrawn at the indicated time points and immediately placed in a boiling water bath for 3 min, followed by centrifugation at 12,000 × *g* for 2 min, and the supernatant was filtered through a 0.22-mm filter and analyzed by HPLC. Reaction samples (10 μl) were injected into an extend C-18-T column (250 by 4.6 cm; Agilent, Santa Clara, CA, U.S.A.) and separated by reversed-phase HPLC at a flow rate of 0.7 ml min^{-1} and a linear gradient from 0 to 20% acetonitrile in the buffer A (10 mm triethylammonium acetate, pH 5.8) during 18 min. Products were detected at 254 nm.

ITC assay.

The binding analysis of Filp was carried out on ITC200 (MicroCal, Northampton, MA, U.S.A.), following the manufacturer's descriptions. In brief, 2 μl of c-di-GMP solution (500 μm) was injected at 2-min intervals via a 60-μl syringe into the sample cell containing 50 μm Filp proteins or the tag protein (20 μM) with constant stirring, and the heat changes accompanying these additions were recorded. The titration experiment was repeated three times, and the data were calibrated with a buffer control and fitted with the single-site model to determine the binding constant (*Kd*) using the MicroCal ORIGIN version 7.0 software.

Construction of gene deletion mutant and complementation analysis.

The mutant was generated using homologous recombination as described previously, by using the suicide vector pK18mobSacB (Schafer et al. 1994). The gene fragment of *filp* was amplified using chromosomal DNA of $PXO99^A$ as template with FilpF/R as primers. The DNA fragments corresponding to downstream and upstream regions of *PXO_02715* were amplified using specific primers 2715LF/LR and 2715RF/RR, respectively. The DNA fragment was digested with corresponding restriction enzymes and ligated to pK18mobsacB. A Gm resistance gene (Gm^r) was amplified from pBBR1MCS plasmid (Kovach et al. 1995) using the primers GmF/GmR, digested with *Nsi*I, and then inserted into *filp* and *PXO_02715* gene fragments carried by pK18mobsacB, resulting in plasmid pKFilpG and pK2715G. pKFilpG and pK2715G were introduced into $PXO99^A$ by electroporation. Potential mutants were selected on PSA plates containing Gm and 10% sucrose. The single colonies that were resistant to Gm but sensitive to Km and high concentrations of sucrose were further confirmed as mutants by PCR analysis.

For complementation analysis, coding regions for the full length of Filp, the PAS-GGDEF-EAL domains (FilpΔR), and REC-PAS-GGDEF domains (FilpΔE) of Filp and PXO_02715 were amplified by primer pairs FilpCF1/FilpCR1, FilpCF2/FilpCR1, FilpCF1/FilpCR2, and 2715C1/2715C2, respectively. FilpΔP fragments were amplified using the primer FilpCF1/FilpCR1 by pCFilpΔP as the template. The FilpΔG fragment was amplified using the primer FilpCF1/FilpCR1 by pCFilpΔG as the template. The PCR fragments were gel purified and cloned to the middle vector pMD18-T (Takara, Tokyo) for sequencing; each fragment was digested from pMD18-T-derived constructs with *Kpn*I/*Hin*dIII and ligated into the vector pBBR1MCS-4, resulting in constructs pBFilp, pBFilpΔR, pBFilpΔP, pBFilpΔG, pBFilpΔE, and pB2715. These constructs were electroporated into Δ*filp* or Δ*PXO_02715* for complementation studies.

RNA isolation and qRT-PCR analysis.

To analyze the expression of T3SS-related genes, bacterial cells of $PXO99^A$ strains were grown in M210 at 28°C to an optical density at 600 nm (OD_{600}) of 0.8. The cells were collected and washed twice with sterilized water and resuspended in XOM2 minimum media at an OD_{600} of 0.8 and cultured for overnight. Then, the cells were collected for RNA extraction. A bacterial RNAout (Tiandz, Beijing) kit was used for total RNA isolation according to the manufacturer's instructions. Total RNA was treated with DNase I (New England Biolabs, Beverly, MA, U.S.A.) to remove any DNA contamination. RNA (5 μg) was used for cDNA synthesis with the random hexanucleotide primers. qPCR analysis was carried out using the gene-specific primers, with cDNAs as template, mixed with the iQTMSYBRR Green Supermix (Bio-Rad). The signals during amplification were detected by the iCycleriQ Real Time Detection System (Bio-Rad). The relative levels of gene expression were determined by using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001), with the DNA gyrase subunit B (*gyrB*) gene as the internal control (Tsuge et al. 2006). Three technical replicates were used each time.

Y2H assay.

Y2H assays were carried out using the Matchmaker Gold yeast two-hybrid system (Clontech, Mountain View, CA, U.S.A.) as described, with minor modifications. The full lengths

of *filp*, *PXO_02715, PXO_02374*, and *PXO_00049* were amplified using the primers FilpYF/FilpYR, 2715YF/2715YR, 2374YF/2374YR, and 49YF/49YR, respectively. PCR products were cloned into the pMD18-T for sequencing. The fragment was then digested from pMD18-T–derived constructs and subcloned into prey vector pGADT7 or bait vector pGBKT7, resulting in pGBKFilp, pGAD2715, pGAD2734, and pGAD49. The constructs were transformed into the *S. cerevisiae* Y187 and Y2HGold strains, respectively. Expressions of fusion proteins in yeast strains were confirmed by Western blotting using anti-cMyc primary antibodies for pGBKT7-derived constructs and anti-HA primary antibodies (Beijing Protein Institute, Beijing) for pGADT7-derived constructs. Pairwise yeast mating between haploid cells containing each pGBKT7 and pGADT7 construct was performed in yeast potato dextrose agar at 28°C for 24 h. The obtained diploids were then selected on double drop-out plates (SD/-Leu/-Trp). Protein interactions were screened on QDO plates (SD/-Leu/-Trp/-Ade/-His). Diploids were suspended in sterile water and four 10-fold serial dilutions were performed before 5 µl of each dilution was placed on the QDO plates. Growth of the diploid yeast cells on QDO plates in 2 days indicated strong interactions between the test proteins.

GST pull-down assay.
For GST pull-down assays, equal amounts of purified proteins were mixed and incubated in the GST-binding columns for 4 h at 4°C in PBS buffer. Eluted proteins were separated on SDS-PAGE 10% gels and transferred to membranes for immunoblotting with either anti-His_6 or anti-GST primary antibodies (Beijing Protein Institute). Primary antibodies were recognized by goat anti-mouse secondary antibodies conjugated with horseradish peroxidase, which were directly visualized by applying the diaminobenzidine substrate (Tiangen Biotech, Beijing).

Virulence test.
The wild-type and mutant strains of $PXO99^A$ were grown for 72 h at 28°C in M210 media with appropriate antibiotics. The cells were collected by centrifugation and resuspended in sterile distilled water at a concentration with OD_{600} of 0.8. The bacterial suspensions were clip inoculated on leaves of approximately 6-week-old rice plants of the susceptible rice variety *O. sativa* subsp. *japonica* under relevant conditions (Ray et al. 2000). Pictures were taken using a digital camera (Canon EOS 550D) and disease lesion lengths were measured 14 days after inoculation.

Motility assay.
Fresh colonies from PSA agar plates were stabbed into plates composed of 0.03% (wt/vol) Bacto Peptone, 0.03% yeast extract, and 0.3% agar for the flagellum-dependent swimming assay (Shen et al. 2001; Yang et al. 2012). Because the sliding motility is highly correlated with secretion of surface-wetting substances and it should be tested under certain nutrient conditions, an SB media plate containing 0.5% (wt/vol) Bacto peptone, 0.5% yeast extract, 0.1% L-glutamic acid, and 0.6% agar was used for sliding assay (Guzzo et al. 2009, 2013; McCarthy et al. 2008; Qi et al. 2012). The inoculated cells were cultured for 4 days at 28°C and examined for bacteria motility away from the inoculated site. The diameter of each swimming or sliding zone was measured and its dimensions were calculated. The numbers represented the average from at least three independent repeats.

EPS production assay.
Bacterial strains were grown in M210 medium for 48 h and the supernatants were collected following centrifugation of cultures at 12,000 × *g* for 10 min. Two volumes of ethanol were added into the supernatants, and the resulting solution was held at –20°C for half an hour. The precipitated EPS was collected by centrifugation and dried overnight at 55°C, and dry weights were recorded as an estimate of EPS (Tang et al. 1996).

Biofilm formation assay.
Log-phase-grown bacteria were diluted to OD_{600} of 0.002, and 5 ml was incubated at 28°C for 72 h in the tubes. Bacterial pellicles were stained by gently pouring off the media, washing twice with water, and staining with 0.1% crystal violet. Tubes were washed and rinsed with water until all unbound dye was removed (An et al. 2010).

HR assay.
To perform HR assays, bacterial cells grown in M210 were resuspended in 10 mM sodium phosphate buffer at a concentration of 10^8 cells/ml (OD_{600} = 0.1) and infiltrated into the leaves of tobacco (*N. benthamiana*) by using a needless syringe. The HR symptoms were observed and photographed at 24 h after inoculation.

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