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A dual role for proline iminopeptidase in the regulation of bacterial motility and host immunity

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SUMMARY

During plant–pathogen interactions, pathogenic bacteria have evolved multiple strategies to cope with the sophisticated defence systems of host plants. Proline iminopeptidase (PIP) is essential to *Xanthomonas campestris* pv. *campestris* (*Xcc*) virulence, and is conserved in many plant-associated bacteria, but its pathogenic mechanism remains unclear. In this study, we found that disruption of *pip* in *Xcc* enhanced its flagella-mediated bacterial motility by decreasing intracellular bis-(3′,5′)-cyclic dimeric guanosine monophosphate (c-di-GMP) levels, whereas overexpression of *pip* in *Xcc* restricted its bacterial motility by elevating c-di-GMP levels. We also found that PIP is a type III secretion system-dependent effector capable of eliciting a hypersensitive response in non-host, but not host plants. When we transformed *pip* into the host plant *Arabidopsis*, higher bacterial titres were observed in *pip*-overexpressing plants relative to wild-type plants after *Xcc* inoculation. The repressive function of PIP on plant immunity was dependent on PIP's enzymatic activity and acted through interference with the salicylic acid (SA) biosynthetic and regulatory genes. Thus, PIP simultaneously regulates two distinct regulatory networks during plant–microbe interactions, i.e. it affects intracellular c-di-GMP levels to coordinate bacterial behaviour, such as motility, and functions as a type III effector translocated into plant cells to suppress plant immunity. Both processes provide bacteria with the regulatory potential to rapidly adapt to complex environments, to utilize limited resources for growth and survival in a cost-efficient manner and to improve the chances of bacterial survival by helping pathogens to inhabit the internal tissues of host plants.

Keywords: bacterial motility, pathogen–host interactions, proline iminopeptidase, repression of host immunity.

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INTRODUCTION

Xanthomonas campestris pv. *campestris* (*Xcc*) is the causal agent of black rot disease in cruciferous plants (Williams, 1980). We have shown previously that the *xccR*/*pip* locus, which encodes the LuxR homologue XccR, regulates the expression of the proline iminopeptidase (PIP, EC 3.4.11.5) gene *pip*, and disruption of either one of the two genes leads to significantly attenuated virulence in *Xcc* (Zhang *et al.*, 2007). Notably, *pip* is genetically conserved in many plant-associated bacteria, and is positioned adjacent to the *luxR* gene in the genome (González and Venturi, 2013). PIP was first identified and characterized in *Escherichia coli* (Sarid *et al.*, 1959). It specifically catalyses the removal of the N-terminal proline residue from peptides or proteins (Cunningham and O'Connor, 1997), and can also remove N-terminal L-alanine or D-alanine from substrates, albeit at lower efficiency (Alonso and Garcia, 1996). In the food industry, PIP is used to debitterize the peptides in cheese processing (Leenhouts *et al.*, 1998; Tan *et al.*, 1993) and in the synthesis of proline-containing dipeptides (Yamamoto *et al.*, 2010, 2011). These studies have elaborated the biochemical properties and biotechnological applications of PIP. However, the molecular mechanism underlying how PIP modulates bacterial virulence is currently unknown.

Flagella and flagellum-mediated cell swimming allow bacteria to approach nutrient sources, invade host cells and escape from host immune system recognition (Chaban *et al.*, 2015; Duan *et al.*, 2013; Josenhans and Suerbaum, 2002; Ottemann and Miller, 1997; Tian *et al.*, 2015). It is believed that flagella-mediated bacterial motility is important for the virulence of pathogens to their host organisms (Broek and Vanderleyden, 1995; Chaban *et al.*, 2015; Duan *et al.*, 2013; Hossain *et al.*, 2005; Josenhans and Suerbaum, 2002). Flagella play important roles throughout the bacterial infection cycle: flagella-mediated swimming motility enables bacteria to reach the host tissues and, once there, they play additional roles, including the promotion of surface adhesion and biofilm formation, which drives bacterial penetration between cell–cell junctions during infection (Chaban *et al.*, 2015; Ottemann and Miller, 1997). *Xcc* possesses a single polar flagellum and more than 40 of its genes are predicted to be

involved in flagellar biogenesis and motility (Yang *et al.*, 2009). The flagellar genes are regulated in a three-tier hierarchy by the RpoD (σ^{70}) housekeeping protein and two alternative sigma factors, RpoN2 (σ^{54}) and FliA (σ^{28}) (Yang *et al.*, 2009). At the top of the regulatory cascade, FleQ functions as a σ^{54}-cognate activator (Hu *et al.*, 2005). During the invasion process, bacterial motility increases the chances of forming surface-attached colonies for bacterial infection (Apel and Surette, 2008). Conversely, bacterial flagellins (FliC), the major structural proteins of Gram-negative flagella, can also trigger defence responses in both plants and animals (Felix *et al.*, 1999; Gewirtz *et al.*, 2001). A flg22 oligopeptide derived from the most conserved region of *Pseudomonas syringae* flagellin, which acts as a pathogen-associated molecular pattern (PAMP), is perceived by the host's FLS2 pattern recognition receptor to invoke PAMP-triggered immunity in plant cells (Zipfel *et al.*, 2004). The bacterial alkaline protease AprA has been found to degrade flagellin monomers for the evasion of host immunity (Pel *et al.*, 2014). The above examples highlight how the fine tuning of flagellar gene expression is crucial for successful bacterial pathogen invasion.

The bacterial second messenger bis-(3′,5′)-cyclic dimeric guanosine monophosphate (c-di-GMP) regulates the transition from the motile planktonic state to sessile community-based behaviour (Wolfe and Visick, 2008). High intracellular levels of c-di-GMP promote sessile lifestyles, such as a biofilm formation lifestyle, whereas low levels of c-di-GMP facilitate motility (Wolfe and Visick, 2008). Cellular c-di-GMP levels are controlled through the opposing activities of diguanylate cyclases with GGDEF domains and phosphodiesterases with EAL or HD-GYP domains, with the former involved in c-di-GMP synthesis and the latter in c-di-GMP degradation (Hengge, 2009; Jenal and Malone, 2006; Römling and Amikam, 2006). Increasing evidence indicates that a range of cellular effectors, such as RpfG, PilZ domain proteins, transcription factors, RNA riboswitches and enzymatically inactive GGDEF and/or EAL domain proteins, interact directly with c-di-GMP and exert diverse influences on bacterial cells (Breaker, 2011; Hengge, 2009; Ryan *et al.*, 2012). FleQ, as a master transcriptional regulator, controls flagella and exopolysaccharide (EPS) gene expression by responding to c-di-GMP (Hickman and Harwood, 2008). The Clp global regulator directly regulates the *fliC* flagellin gene of *Xcc*, and a large quantity of c-di-GMP binds specifically to Clp to abolish the interaction between Clp and the *fliC* promoter, thereby reducing bacterial motility (Lee *et al.*, 2003; Tao *et al.*, 2010).

The bacterial type III secretion system (T3SS) is an injectisome evolutionarily related to the bacterial flagellum (Notti *et al.*, 2015). Many Gram-negative plant- and animal-pathogenic bacteria exploit the evolutionarily conserved T3SS to overcome host defences by the direct delivery of effectors to the host cell interior (He *et al.*, 2004). It is interesting to note that c-di-GMP plays fundamental roles in the control of multiple important bacterial export pathways. High levels of c-di-GMP lead to the repression of *Pseudomonas aeruginosa* T3SS expression (Moscoso *et al.*, 2011) and the inhibition of ATPase activity through direct allosteric control of the export of ATPase proteins by binding with c-di-GMP (Trampari *et al.*, 2015). Type III effectors from plant-pathogenic bacteria have multiple targets in plants and interfere with broad cellular pathways, including host immune responses, cytoskeletal dynamics, proteasome-dependent protein degradation and phytohormone signalling (Buttner, 2016). About 40 T3SS effectors have been identified in xanthomonads, and a few have been found to interact with host-specific factors to exert virulence functions or to trigger immune responses (Gürlebeck *et al.*, 2006; White *et al.*, 2009). Salicylic acid (SA) induction in plants is a general resistance mechanism used against pathogen infection (Vlot *et al.*, 2009). The prevention of SA accumulation leads to disease susceptibility (Gaffney *et al.*, 1993), whereas SA treatment confers resistance to a variety of biotrophic pathogens (Ryals *et al.*, 1996; White, 1979). SA production in plants occurs mainly through the isochorismate pathway (Chen *et al.*, 2009). In *Arabidopsis*, isochorismate synthase 1 (ICS1), a crucial enzyme in the SA biosynthetic pathway, is induced by biotic and abiotic stresses (Wildermuth *et al.*, 2001). SA activates defence responses through its downstream component, non-expressor of pathogenesis-related genes 1 (NPR1), which functions as a key regulator of the SA signalling pathway (Dong, 2004). EDS1 (enhanced disease susceptibility 1) contributes to post-invasive non-host resistance, as well as to SA-dependent and SA-independent defence signalling, in incompatible host–pathogen interactions (Bartsch *et al.*, 2006; Lipka *et al.*, 2008; Wiermer *et al.*, 2005). The EDS1/PAD4 (phytoalexin deficient 4) complex acts upstream of SA signalling, and contributes to the positive feedback loop for SA accumulation (Feys *et al.*, 2001). To counteract plant resistance, the type III effector can suppress plant innate immunity by the up-regulation of hormones that are antagonistic to SA, such as the *P. syringae* AvrPtoB effector, which triggers elevated foliar abscisic acid (ABA) levels to suppress callose deposition and increase plant susceptibility to pathogens (de Torres *et al.*, 2006). The *X. campestris* pv. *vesicatoria* effector XopJ interacts with the RPT6 plant proteasomal subunit to interfere with SA-dependent defence responses by the inhibition of proteasome activity (Ustun *et al.*, 2013). The chloroplast-localized J domain-containing HopI1 causes chloroplast thylakoid structure remodelling by interaction with Hsp70, thereby suppressing SA accumulation (Jelenska *et al.*, 2007).

Here, we show that the disruption of *pip* in *Xcc* results in increased bacterial motility and the activation of multiple flagellar genes. The reduced level of c-di-GMP observed in the *pip* mutant implies that PIP has a regulatory effect on c-di-GMP synthesis or catabolism, which may be advantageous for bacterial communication and group behaviour. We also found that, as a type III effector, PIP can modulate the host plant immune system by the

repression of the SA signalling pathway. Our findings offer an insight into the role of PIP by showing that it not only modulates bacterial physiological processes, but also controls the environmental niches for successful establishment of infection by bacterial pathogens.

RESULTS

Key amino acid residues for PIP enzymatic activity

We searched the MEROPS database, an integrated source of information on peptidases (Rawlings, 2016), and found that PIP belongs to the prolyl aminopeptidase S33 family, which preferentially releases an N-terminal proline residue from peptides. Three residues (S106, D246 and H273) were predicted to comprise the enzymatic active site of PIP (Fig. 1A). To verify the contribution of these key residues to enzymatic activity, PIP variants, each with a single alanine amino acid substitution, were expressed from plasmid pET30a in *E. coli* BL21 (DE3). The enzyme activity assays showed that the alteration of any of these three residues almost completely abolished the enzyme's proline residue-releasing ability from the substrate (Fig. 1B), demonstrating that these three amino acids are equally essential for the enzymatic activity of PIP.

Disruption of PIP leading to increased *Xcc* motility

Our previous work has shown that PIP is important for *Xcc* virulence and that *pip* expression is induced *in planta* (Zhang *et al.*, 2007). However, how PIP affects the bacterial infection process remains unknown. Hence, we first tested the swimming phenotype in *Xcc* strains on XOLN medium (Fu and Tseng, 1990) (plant environment mimicking medium) motility plates (0.3% agar). The *pip*-deficient mutant (*pip*$^-$) strain showed significantly higher swimming motility, whereas the *pip*$^-$/pLAFR3-*pip* strain restored the motility to the wild-type level (Fig. 2). Plasmids pLAFR3-*pip*^{S106A}, pLAFR3-*pip*^{D246A} and pLAFR3-*pip*^{H273A} were separately transformed into the *pip*-deficient mutant, but the bacterial motility was not restored, indicating that PIP-modulated bacterial swimming ability is dependent on PIP enzymatic activity (Fig. 2). We further examined the expression of the flagella-associated genes listed in Table S1 (see Supporting Information). Real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR) assays showed that a lack of *pip* led to an extensive induction of flagellum genes in XOLN medium. Of the three classes of flagella-related genes, half of the tested genes were significantly increased in the *pip*-deficient mutant. Class II genes, such as *fleN*, *fliA* (σ^{28}) and *flgH*, were significantly increased by 5.42-fold, 28.93-fold and 84.21-fold, respectively (Fig. 3A–C). The expression levels of *fliQ* and *flgB* genes were increased even more, by as much as 141.06-fold and 130.84-fold, respectively (Fig. 3D,E), whereas the levels of the other tested flagella-related genes remained unchanged (Table S1). The levels of Class III genes, such as *fliC* and *fliD*, increased by 49.43-fold and 9.45-fold, respectively (Fig. 3F,G). The expression levels of the same genes in the *pip*$^-$/pLAFR3-*pip* strain were complemented or increased compared with that in wild-type *Xcc* 8004, except for *fleN* (Fig. 3). In contrast, *pip* overexpression in *Xcc* 8004 restricted gene expression to even lower levels compared with the *pip*$^-$/pLAFR3-*pip* strain (Fig. 3). These results suggest that PIP is a repressor or negative regulator of flagellum-mediated motility.

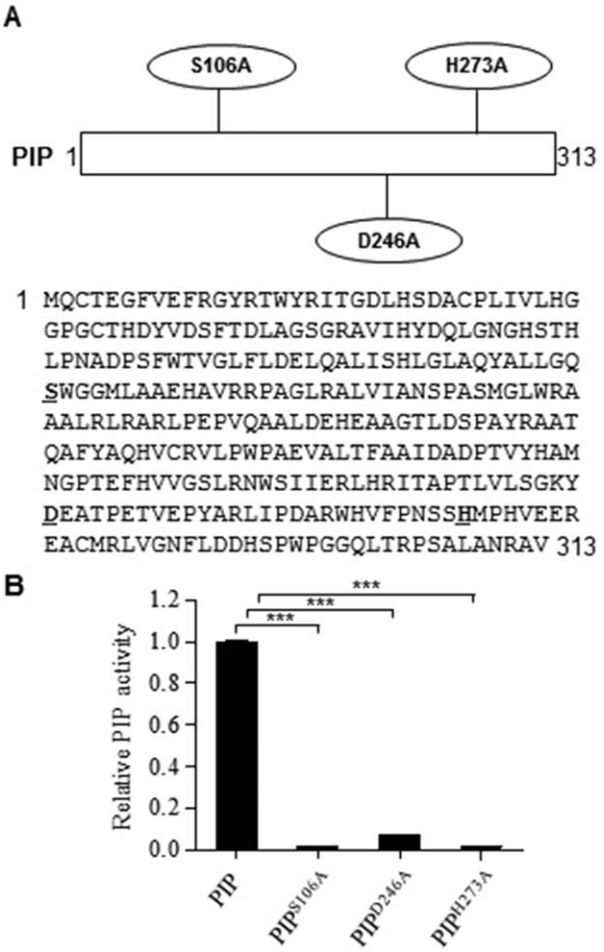

Fig. 1 Key amino acid residues for proline iminopeptidase (PIP) enzymatic activity. (A) The amino acid sequence of PIP. Three amino acid residues at the PIP enzyme active site are shown with oval borders and with underlined bold letters in the sequence. The key residues, including a serine residue, a histidine residue and an aspartic acid residue, were mutated to an alanine residue separately. (B) Relative enzymatic activity of PIP and derived mutated proteins. The recombinant proteins were expressed and purified from *Escherichia coli*. The enzymatic activities of PIP were determined using L-proline *p*-nitroanilide trifluoroacetate salt (PPNA) as a substrate. Data are shown as the mean ± SEM (standard error of the mean) of three independent experiments. Statistical significance was determined using a one-way analysis of variance (ANOVA; $***P < 0.0001$).

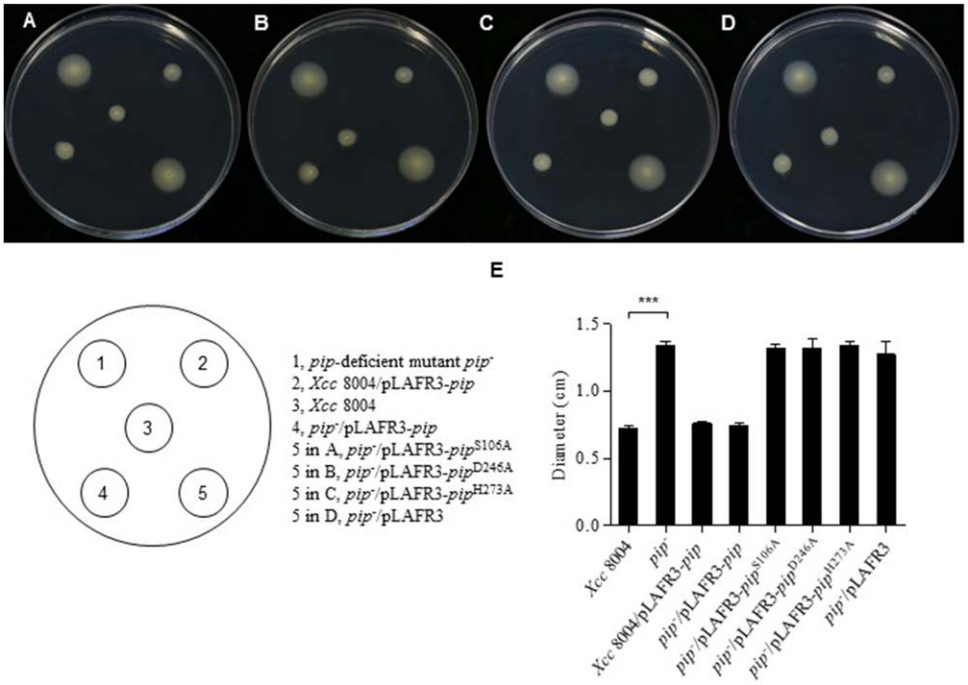

Fig. 2 Regulation of *Xanthomonas campestris* pv. *campestris* (*Xcc*) motility by *pip*. (A–D) Motility phenotype of wild-type *Xcc* 8004, *pip*⁻, *Xcc* 8004/pLAFR3-*pip* and *pip*⁻/pLAFR3-*pip* strains on XOLN swimming plates. Photographs were taken at 48 h after incubation at 28 °C. (E) Bars represent the mean diameters of the migration zones of each strain. Error bar represents SEM (standard error of the mean) for at least three replicates with statistical analysis using one-way analysis of variance (ANOVA; $***P < 0.0001$).

Intracellular c-di-GMP level modulated by PIP

The important second messenger c-di-GMP has been shown to control a variety of biological processes, including motility, in many bacteria (Römling *et al.*, 2013). To explore whether PIP-controlled bacterial motility is correlated with c-di-GMP content, the intracellular levels of c-di-GMP were measured by high-pressure liquid chromatography-tandem mass spectrometry (HPLC-MS/MS). As shown in Fig. 4, the *pip*-deficient mutant had a lower level of c-di-GMP than the wild-type *Xcc* 8004. The c-di-GMP level was markedly elevated by introducing pLAFR3-*pip* into the *pip*-deficient mutant (Fig. 4). Overexpression of *pip* in *Xcc* 8004 resulted in an even higher concentration of intracellular c-di-GMP, and it was 2.5-fold higher than that of the wild-type strain (Fig. 4). These results are consistent with those of previous studies reporting lower levels of c-di-GMP-promoted bacterial motility (Jenal and Malone, 2006), and high levels of c-di-GMP repressing flagellar assembly by binding to FleQ (Ha and O'Toole, 2015). We propose that PIP may modulate bacterial motility via the c-di-GMP signalling pathway.

PIP as a new type III effector

In plant-pathogenic bacteria, the T3SS is encoded by *hrp* (*h*ypersensitive *r*esponse and *p*athogenicity) and *hrc* (*h*ypersensitive *r*esponse and *c*onserved) gene clusters (Lindgren *et al.*, 1986). HrcV, the conserved structural component of the T3SS apparatus, is involved in substrate docking and is responsible for the secretion of effector proteins (Hartmann and Büttner, 2013). We constructed an in-frame deletion mutant of *hrcV* (Δ*hrcV*) impaired in its T3SS function. To investigate whether PIP can be delivered into the plant cytoplasm, we constructed C-terminal translational calmodulin-dependent adenylate cyclase (Cya) fusions to PIP (1–100 amino acids). The kinetics of cyclic adenosine monophosphate (cAMP) accumulation in plant leaves were examined following inoculation with *Xcc* 8004 or Δ*hrcV* strains expressing $PIP_{(1–100)}$-Cya. As additional controls, plants were inoculated with *Xcc* 8004 or Δ*hrcV* containing an empty vector (pLAFR3), *Xcc* 8004/pLAFR3-$eGFP_{(1–244)}$-*cya* as a negative control and *Xcc* 8004/pLAFR3-$AvrBS2_{(1–98)}$-*cya* as a positive control. As shown in Fig. 5A,B, although the Cya-tagged fusion proteins were detected in the cell lysates of *Xcc* 8004/pLAFR3-$pip_{(1–100)}$-*cya*, *Xcc* 8004/pLAFR3-$AvrBS2_{(1–98)}$-*cya*, *Xcc* 8004/pLAFR3-$eGFP_{(1–244)}$-*cya* and Δ*hrcV*/pLAFR3-$pip_{(1–100)}$-*cya* strains by western blotting, cAMP accumulation was detected only in the *Xcc* 8004/pLAFR3-$pip_{(1–100)}$-*cya* and *Xcc* 8004/pLAFR3-$AvrBS2_{(1–98)}$-*cya* inoculated plants, consistent with the western blotting results in the culture supernatants (Fig. 5B). These data indicate that PIP secretion and delivery into plant cells depend on the T3SS. Some bacterial effectors can lead to a hypersensitive reaction (HR) on non-host plants (Mysore and Ryu, 2004). To further investigate the effect of PIP in different plants, the purified PIP protein was allowed to infiltrate plant leaves. The lesions caused by cell death were observed at 2 days post-infiltration in non-host tobacco leaves, and the HR lesion became more severe with greater amounts of PIP protein (Fig. 6A). In contrast, PIP protein infiltration did not induce HR lesions in the leaves of host cabbage plants (Fig. 6C,D). Similarly, when using different strains to inoculate tobacco leaves, we found that the *pip*-overexpressing (*Xcc* 8004/pLAFR3-*pip*) strain caused

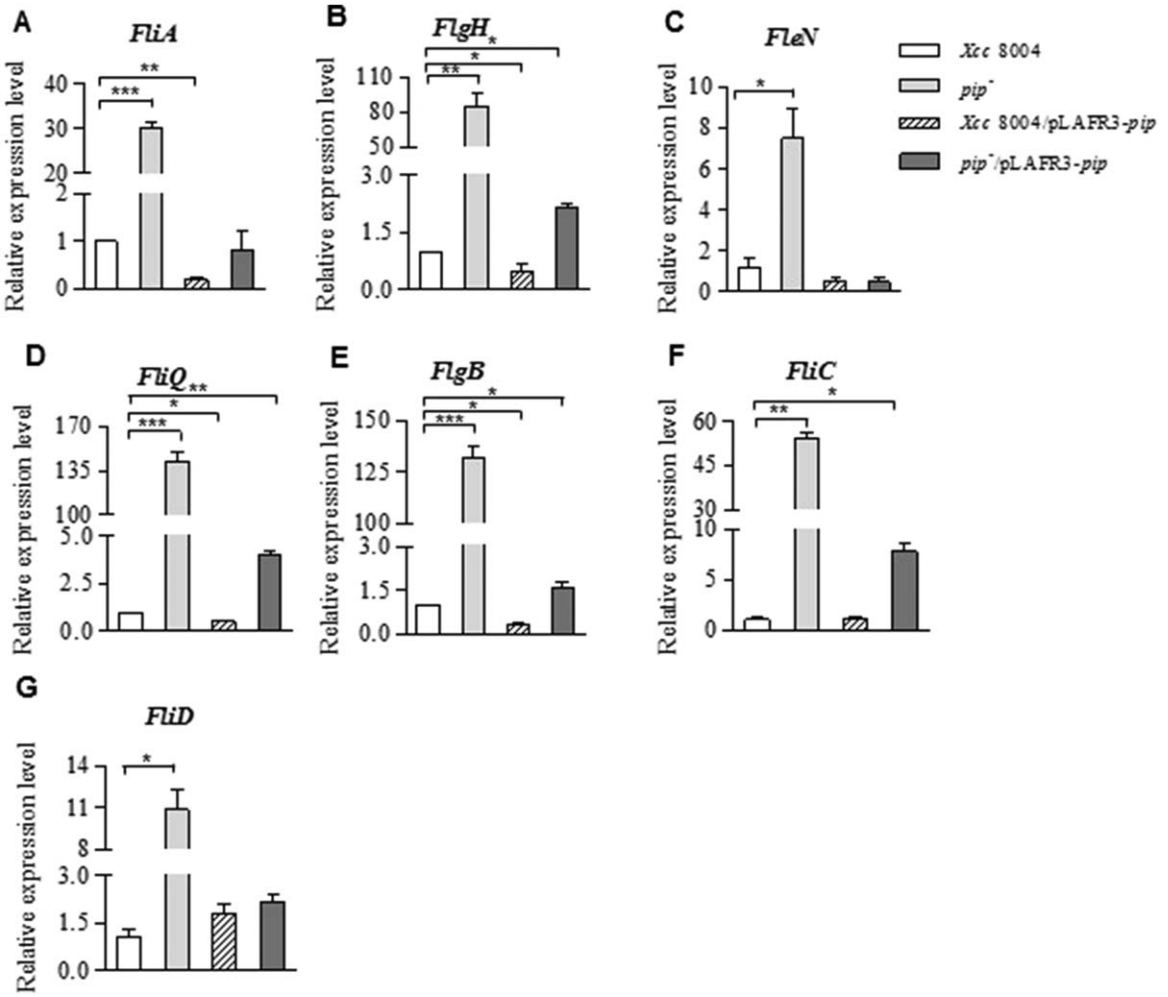

Fig. 3 Proline iminopeptidase (PIP) affects flagellar gene expression. (A–G) Relative expression levels of flagellar genes in wild-type *Xcc* 8004, *pip*⁻, *Xcc* 8004/pLAFR3-*pip* and *pip*⁻/pLAFR3-*pip* strains determined by quantitative reverse transcription-polymerase chain reaction (RT-PCR). The expression level of each gene in *Xanthomonas campestris* pv. *campestris* (*Xcc*) 8004 is indicated as 1.0. 16S rRNA was used as the internal reference. Error bar represents SEM (standard error of the mean) for at least three replicates with statistical analysis using one-way analysis of variance (ANOVA; $***P < 0.0001$, $**P < 0.001$, $*P < 0.05$).

HR lesions more quickly than did the wild-type *Xcc* 8004 and the *pip*-deficient mutant (Fig. 6E). Interestingly, PIP enzymatic activity was dispensable for the elicitation of cell death in non-host plants because the same phenotypes were observed when wild-type PIP or the PIP^{S106A} protein (one of the three enzymatic activity-deficient proteins was selected to conduct the assays) was injected into tobacco leaves (Fig. 6A,B). Both PIP and PIP^{S106A} failed to elicit cell death in host cabbage plant leaves (Fig. 6C,D), indicating that PIP of *Xcc* escaped recognition by the host plant immune system, and that this helped to boost bacterial virulence.

PIP interferes with the SA signalling pathway to benefit bacterial growth

Transgenic *Arabidopsis* plants that express wild-type PIP or PIP^{S106A} were obtained to determine whether the enzymatic activity of PIP influences host plant resistance. Both the wild-type and S106A mutated PIP protein were detectable in transgenic *Arabidopsis* plants on the western blots (Fig. 7A). The enzymatic activity of PIP in the leaves of *pip* transgenic plants was much higher than that in those of the wild-type Col-0 and pip^{S106A} transgenic plants (Fig. 7B). To measure bacterial growth *in planta*, 4-week-old *Arabidopsis* seedlings were infiltrated by *Xcc* cells, and the number of colony-forming units (CFUs) recovered from the infected leaves was counted at 72 h post-inoculation (hpi) (Fig. 7C). The number of CFUs recovered from *pip* transgenic *Arabidopsis* was log 1.06-fold higher than the number of CFUs recovered from wild-type plants (Fig. 7C), whereas the number of CFUs in the pip^{S106A} transgenic plants did not differ from that of the wild-type plants. These data show that the *pip* transgenic *Arabidopsis* plants were more susceptible than their wild-type counterparts to *Xcc*, and that PIP

enzymatic activity played an important role in the repression of host resistance.

Whether plant–microbial interactions are beneficial or detrimental to plants depends largely on the phytohormone homeostasis in the plant (Denance *et al.*, 2013). To investigate further whether plant immunity is influenced by PIP, gene expression in the SA signalling pathway was measured by real-time quantitative RT-PCR. As shown in Fig. 8, the transcription levels of *ICS1*, *NPR1*, *EDS1* and *PAD4* in PIP-expressing plants were 14.12-, 2.8-, 2.3- and 1.2-fold lower, respectively, than those in wild-type plants at 24 h after *Xcc* infection. The *ICS1* and *NPR1* expression levels in PIP^{S106A}-expressing plants were comparable with those in wild-type plants, and the levels of *EDS1* and *PAD4* were 64% and 48% of the wild-type levels, respectively. We also measured SA levels in *Arabidopsis* plants using HPLC-MS/MS. After *Xcc* infection, the *pip* transgenic plants accumulated lower levels of free SA than the wild-type plants (reduced to 67.2%), whereas the PIP activity-deficient (pip^{S106A}) plants showed unrestricted SA accumulation (Fig. 8E). These data indicate that bacterial PIP interferes with the SA signalling pathway of the host plant, which is beneficial for bacterial infection.

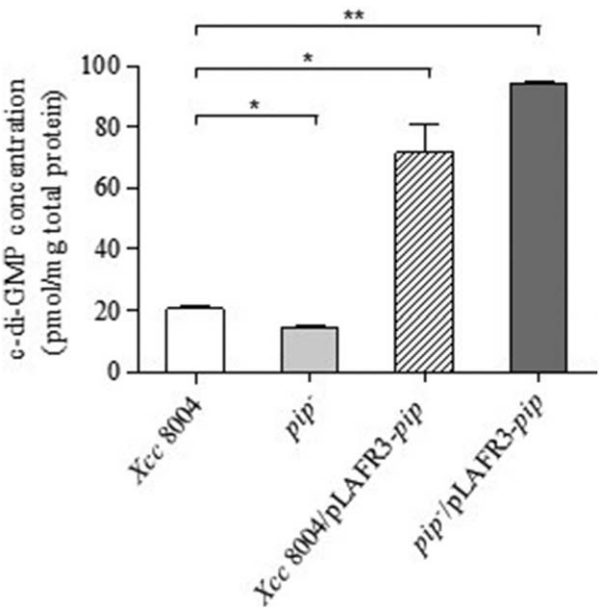

Fig. 4 Bacterial intracellular bis-(3′,5′)-cyclic dimeric guanosine monophosphate (c-di-GMP) levels are correlated with proline iminopeptidase (PIP) expression. c-di-GMP was extracted from the bacterial cells of each strain at an optical density at 600 nm (OD_{600}) of 0.2, and quantified using high-pressure liquid chromatography-tandem mass spectrometry (HPLC-MS/MS). Values were normalized by total protein contents measured by Bradford protein assay. The data represent the averages from three replicates; error bars indicate SEM (standard error of the mean). Statistical analysis was performed using one-way analysis of variance (ANOVA; $*P < 0.05$, $**P < 0.001$).

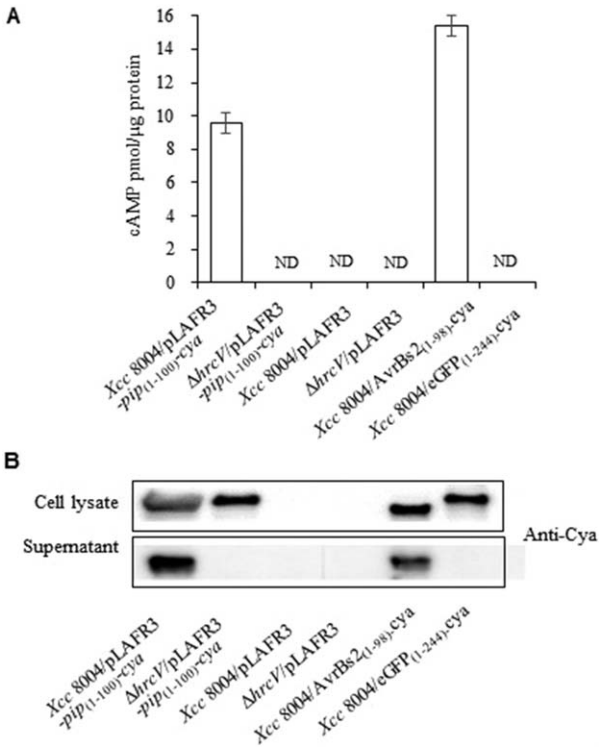

Fig. 5 Proline iminopeptidase (PIP) is a new type III effector. (A) The calmodulin-dependent adenylate cyclase (Cya) activity was determined by testing cyclic adenosine monophosphate (cAMP) concentration after 10 h of infiltration of bacterial strains into *Arabidopsis* plant leaves. Values were normalized by the protein contents in each sample. Δ*hrcV* is a type III secretion-deficient mutant. The data were repeated at least three times. (B) The expression assay of Cya-tagged fusion protein in bacterial cells and culture supernatants through western blotting with anti-Cya mouse monoclonal antibody. ND, not detected.

DISCUSSION

During co-evolutionary processes, plant pathogens have evolved various strategies to counteract plant immune systems. The role of the T3SS and bacterial flagella in pathogenesis is well established, but having one protein involved in both systems is uncommon in bacteria. Our study has revealed that PIP not only modulates bacterial motility, but also acts as a type III effector capable of inhibiting plant immunity. We propose that PIP may exert its effects during the early stages of infection with *Xcc*, but the mechanism underlying how the two systems are coordinated is still unknown.

PIPs are evolutionarily conserved in many plant-associated bacteria and are involved in bacterial pathogenesis (González and Venturi, 2013; Zhang *et al.*, 2007), but their respective mechanisms of action have not been established. Here, we observed that the *pip*-deficient mutant showed much higher swimming ability than the wild-type strain on XOLN medium motility plates (Fig. 2). XOLN is a basal salt medium that mimics a low-nutrient

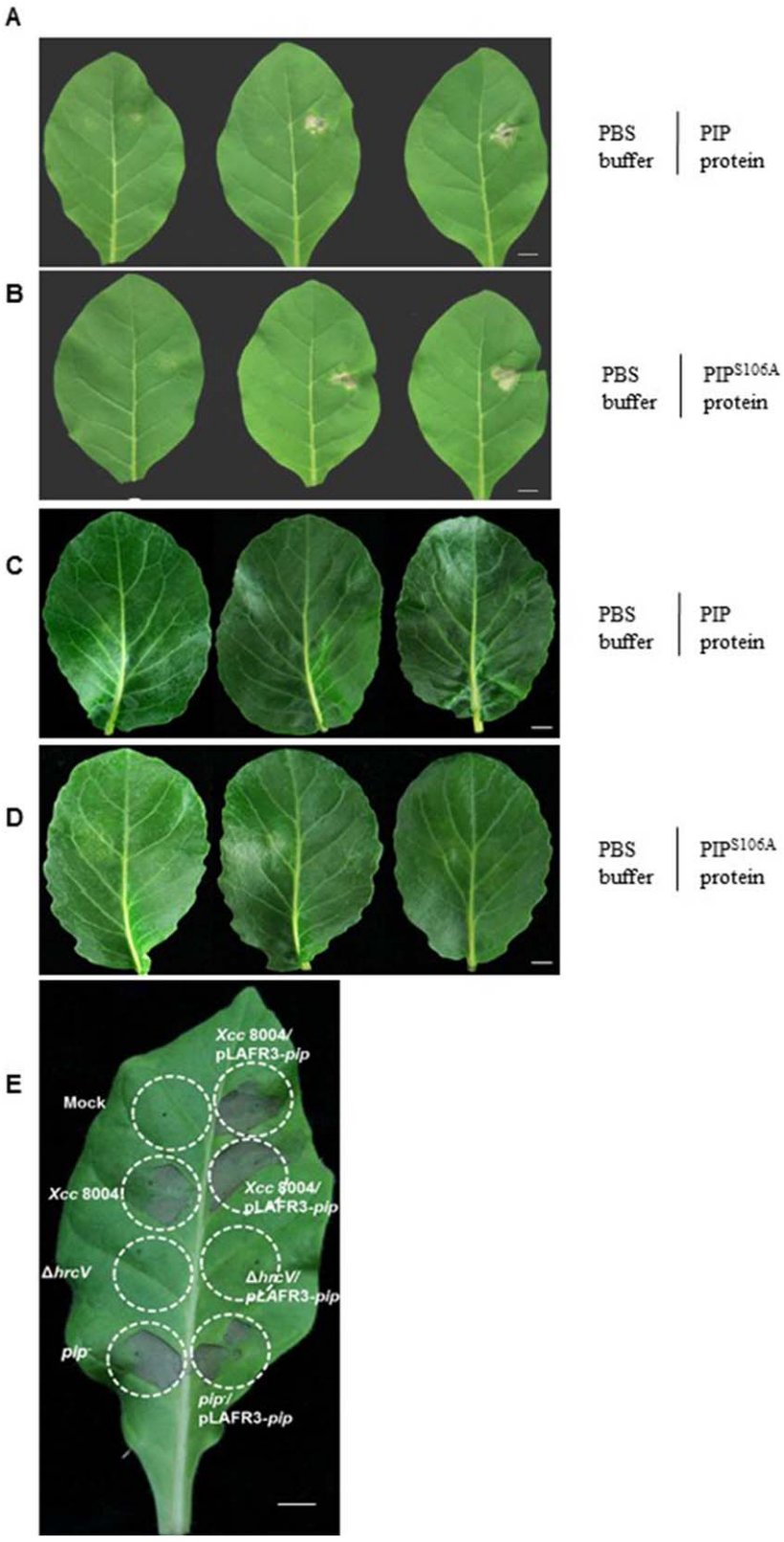

Fig. 6 Cell death and hypersensitive response (HR) observation in non-host plant tobacco and host plant cabbage leaves. Purified proline iminopeptidase (PIP) protein (A) and PIP^{S106A} protein (B) were injected into non-host leaves and led to cell death symptoms after 2 days. Left halves: phosphate-buffered saline (PBS) treatment as the negative control; right halves: different concentrations of PIP protein (A) or PIP^{S106A} protein (B) treatment for cell death observation. From left to right: 1 mg/mL, 5 mg/mL and 10 mg/mL of protein. PIP protein (C) and PIP^{S106A} protein (D) could not induce cell death in cabbage leaves, and the protein inocula were the same as in (A) and (B). (E) HR assay of different bacterial strains with a bacterial titre of 10^9 colony-forming units (CFU)/mL in tobacco leaves. Mock: 10 mM $MgCl_2$ buffer. Scale bar, 1 cm.

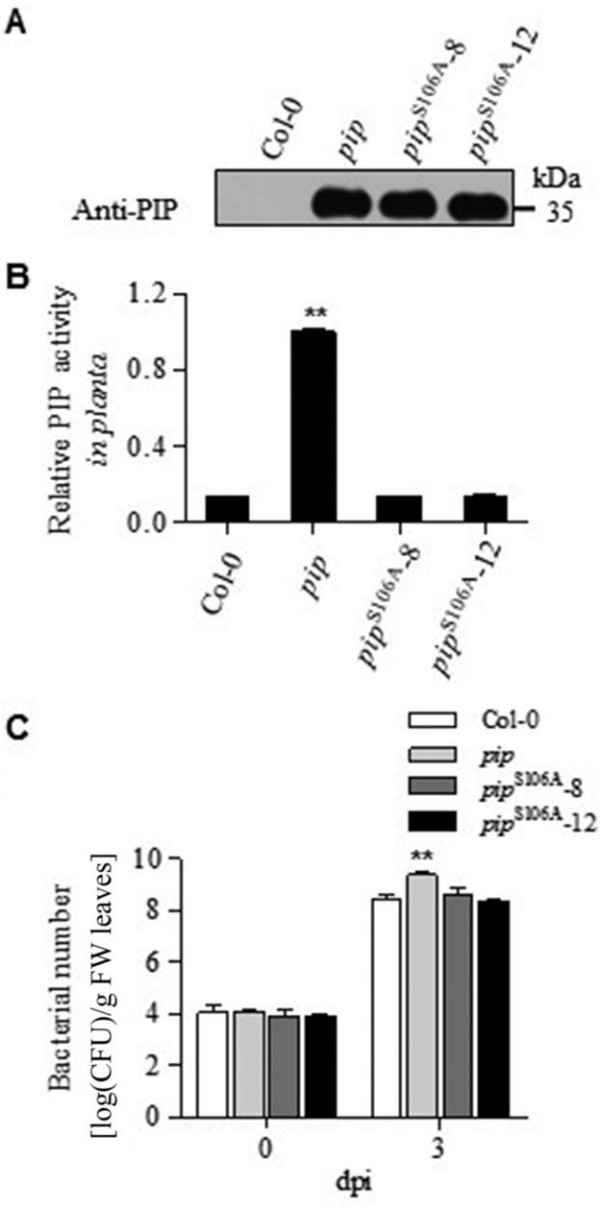

Fig. 7 Proline iminopeptidase (PIP) activity is essential for *Xanthomonas campestris* pv. *campestris* (*Xcc*) pathogenicity. (A) Western blot assay of PIP expression in wild-type Col-0 and *pip* or *pip*^{S106A} transgenic plants; PIP^{S106A}-8 and PIP^{S106A}-12 represent two independent transgenic lines. (B) Relative PIP enzymatic activities were determined by normalization to the level in *pip* transgenic plants ($n = 9$). (C) Bacterial populations in Col-0 and different transgenic plants ($n = 5$). Statistical analysis was a one-way analysis of variance (ANOVA) (**$P < 0.001$), and the error bar represents SEM (standard error of the mean). dpi, days post-inoculation; FW, fresh weight.

environment in plants (Fu and Tseng, 1990). We found that the expression levels of nine flagellar genes in the three-level hierarchical regulatory cascade, including the *fliA* (σ^{28}) regulatory factor, for example, significantly increased in the *pip*-deficient mutant (Fig. 3A). With regard to the flagellar genes, when compared with the wild-type strain, the expression of *fliQ* and *flgB* increased 100-fold (Fig. 3D,E). Conversely, the majority of the flagellar genes tested showed decreased expression levels in *Xcc* 8004/pLAFR3-*pip* compared with the wild-type strain (Figs 2 and 3), although bacterial motility showed no significant differences between them. One possible reason may be that the catalytic activity of PIP in the wild-type strain is saturated for the control of bacterial movement, and so excessive PIP in the *Xcc* 8004/pLAFR3-*pip* strain has no further effect on motility. Because *pip* expression was induced when *Xcc* 8004 infiltrated host plant leaves (Zhang *et al.*, 2007), we speculate that this may cause reduced bacterial motility in the host plant. Co-regulation of flagellar genes by PIP is helpful for the control of bacterial motility behaviour. Flagella-mediated motility enables bacteria to reach optimal niches, but becomes less essential when the pathogen contacts the host tissues (Chaban *et al.*, 2015; Josenhans and Suerbaum, 2002). Consequently, the motility apparatus is subject to strict energy-saving control during pathogen–host interaction. We also noticed that the expression of *fliC* (encoding flagellin) was regulated by PIP (Fig. 3F). Flagellin molecules are the main structural proteins assembled into flagella and can be secreted into the culture medium (Komoriya *et al.*, 1999). A highly conserved 22-amino-acid peptide (flg22) region of flagellin (which serves as a PAMP) was responsible for detectable elicitation of the *Arabidopsis* defence responses mediated by the plant's FLS2 (Sun *et al.*, 2006). In some *Xcc* strains, the variable flg22 peptides elicit resistance responses in host plant leaves (Sun *et al.*, 2006); thus, restriction of bacterial PAMP levels may circumvent dramatic defence responses in plants. In *Xcc* 8004, the flagellin flg22 domain amino acid sequence (QQLSSGKRITSASVDAAGLAIS) is identical to that of the *Xcc* B186 strain (Sun *et al.*, 2006), which has no defence-eliciting activity on *Arabidopsis* Col-0.

c-di-GMP, a novel global bacterial second messenger, is involved in motility, EPS production, biofilm formation and virulence, and its metabolism is controlled by many proteins with GGDEF, HD-GYP and EAL domains (Fouhy *et al.*, 2006; Ryan and Dow, 2010). Consistent with previous findings, the lack of PIP and the increased intracellular c-di-GMP level in the reduced mobility *pip*-overexpressing strain led to decreased c-di-GMP levels in bacterial cells with increased motility compared with the wild-type strain (Figs 2 and 4).We propose that *pip* may directly or indirectly affect c-di-GMP metabolism and alter bacterial motility, but the interacting target of PIP is unclear. In our assay, the induction of flagella-associated genes was negatively correlated with the intracellular content of c-di-GMP in the different strains (Figs 3 and 4), suggesting that PIP may affect flagellar gene expression via the c-di-GMP signalling pathway. The expression of multiple flagella genes is controlled by the c-di-GMP-binding transcriptional regulator FleQ (Hickman and Harwood, 2008), and so it is also possible

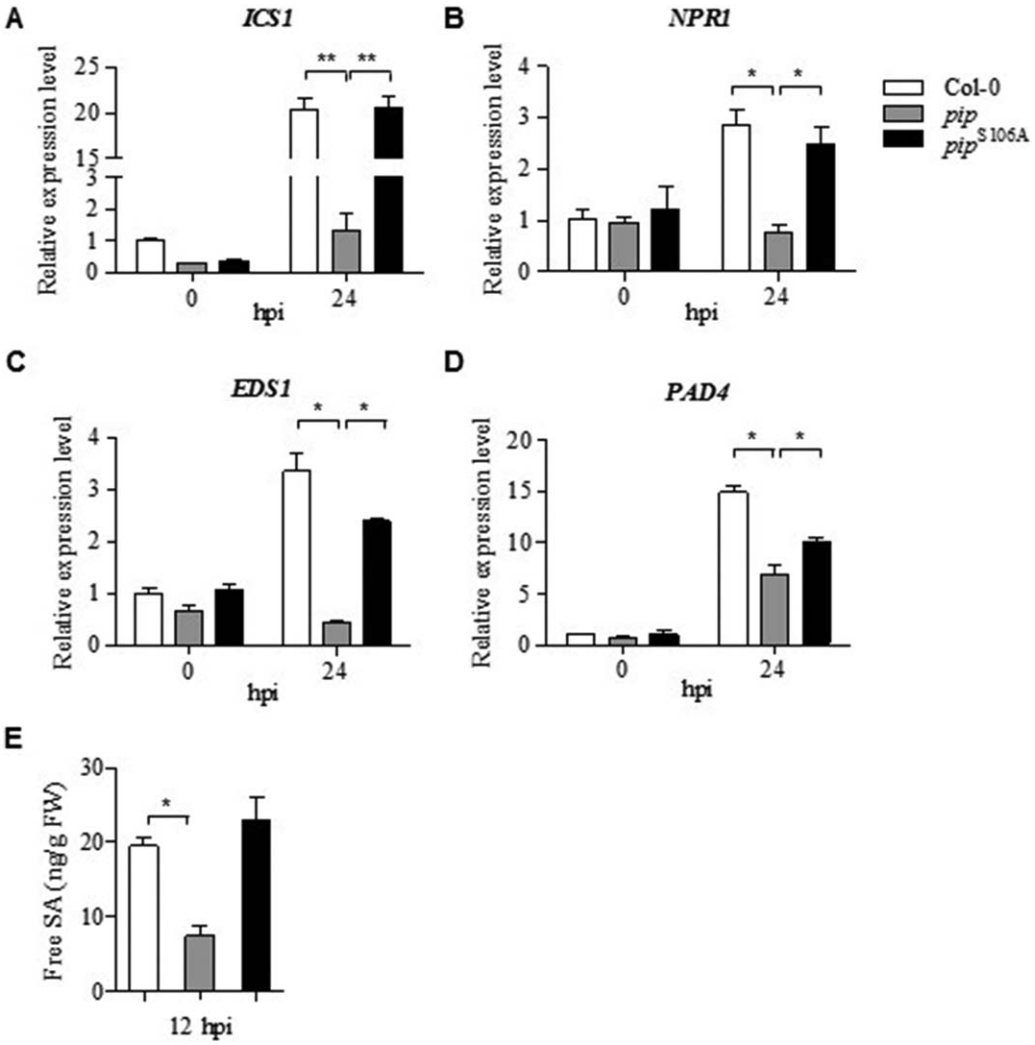

Fig. 8 Proline iminopeptidase (PIP) interference with the salicylic acid (SA) signalling pathway in host *Arabidopsis* plants. Quantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis of SA biogenesis and signalling pathway gene expression in wild-type Col-0, transgenic *pip* and pip^{S106A} plants, including (A) *ICS1*, (B) *NPR1*, (C) *EDS1* and (D) *PAD4*, with *actin2* as the internal reference. (E) Free SA levels in *Xanthomonas campestris* pv. *campestris* (*Xcc*)-infected plants measured by liquid chromatography-tandem mass spectrometry (LC-MC/MS). Error bar represents SEM (standard error of the mean) for at least three replicates with statistical analysis using a one-way analysis of variance (ANOVA; $^{**}P < 0.001$, $^{*}P < 0.05$). FW, fresh weight; hpi, hours post-inoculation.

that the altered levels of c-di-GMP mediated by PIP may occur through FleQ to influence bacterial motility. We found that PIP exerts its modulating function on bacterial motility via its enzymatic activity (Figs 3 and 4), and it is highly likely that PIP modulates unidentified targets at the post-translational level. An important feature of c-di-GMP regulation is its ability to control critical lifestyle transitions, such as motile–sessile transitions. We propose that PIP may contribute to the modulation of bacterial motility when *Xcc* contacts plant tissues.

Pathogenic bacteria deliver virulence effectors into the plant cytoplasm mainly through bacterial secretion systems to ensure their destructive function (Gerlach and Hensel, 2007). In Gram-negative bacteria, type III effectors are the most important virulence factors for the reprogramming of host cellular functions for bacterial benefit (He, 1998). Surprisingly, PIP was verified as a new T3SS effector by the detection of PIP secretion in culture supernatants and when the PIP-Cya fusion protein triggered cAMP accumulation in an *hrcV*-dependent manner in the host plants (Fig. 5). HrpX is a global regulator of T3SS gene expression (Koebnik *et al.*, 2006; Wengelnik and Bonas, 1996). Our previous results have shown that both HrpX and XccR are responsible for *pip* expression in host cabbage plants (Zhang *et al.*, 2007, 2012), indicating that PIP may respond to different environmental signals to co-ordinate bacterial behaviour. Intriguingly, purified PIP elicited cell death in non-host tobacco plant leaves, but not in host cabbage leaves, and the phenotypes were not related to PIP

enzymatic activity (Fig. 6). It is likely that the intact structure of the PIP protein, rather than its enzyme activity *per se*, is recognized by non-host plants. Generally, type III effectors function primarily inside host cells when translocated by bacteria. The purified PIP protein triggers cell death on tobacco leaves, suggesting that it could also be recognized outside the plant cells. It is possible that the PIP protein could be released from the dead or broken bacterial cells when bacteria enter the plant apoplast.

Successful pathogens have evolved diverse strategies to subvert the multilayered plant immune system and exploit their respective host plants (Boller and He, 2009). The prevention of the accumulation of SA, a key immune hormone, is an important strategy employed by plant pathogens (Kumar, 2014), as reducing its levels in plants leads to disease susceptibility (Gaffney *et al.*, 1993). We introduced *pip* and pip^{S106A} into *Arabidopsis* plants by *Agrobacterium*-mediated transformation (floral dipping). Plants with PIP enzymatic activity were more susceptible to *Xcc* infection, whereas pip^{S106A} plants were not (Fig. 7C). We observed that PIP reduced the expression of SA biosynthesis-related (*ICS1*) and resistance response-related (*EDS1*, *PAD4* and *NPR1*) transcripts in *pip* transgenic plants after *Xcc* infection, a finding consistent with lower SA accumulation (Fig. 8). These results show that *Xcc* PIP is involved in the repression of SA accumulation and interference with the SA signalling pathway to favour disease.

This work reveals the functionality of PIP at different locations in its host: by involvement in flagella-associated motility regulation or as a virulence effector capable of overcoming the plant immune system by interfering with its SA signalling pathway. The fine tuning of *pip* gene expression at different stages of plant infection suggests the need for hierarchical and temporal control over its translocation. PIP can remove N-terminal prolines from peptides or proteins (Cunningham and O'Connor, 1997), making such substrates potentially modifiable by PIP. However, PIP triggers HR in non-host plant leaves in the absence of enzymatic activity, and so the possibility cannot be excluded that the targets of PIP are not its enzymatic substrates. Exploring these substrates in bacteria and plants will augment our understanding of the molecular mechanisms involved in plant–pathogen interactions. These results will provide a strategy with which to defeat pathogen invasion or enhance plant resistance to improve crop quality.

EXPERIMENTAL PROCEDURES

Bacterial strains and growth conditions

The bacterial strains, plasmids and primers used in this study are shown in Table S2 (see Supporting Information). *Xcc* 8004 (Daniels *et al.*, 1984) and derivative strains were cultured at 28 °C in NYG rich medium (Daniels *et al.*, 1984) and XOLN medium (0.7 g/L K_2HPO_4, 0.2 g/L KH_2PO_4, 1.0 g/L $(NH_4)_2SO_4$, 0.1 g/L $MgCl_2 \bullet 6H_2O$, 0.01 g/L $FeSO_4 \bullet 7H_2O$, 0.001 g/L $MnCl_2$, 0.625 g/L yeast extract, 0.625 g/L tryptone, pH 7.5) (Fu and Tseng, 1990). *Escherichia coli* and *Agrobacterium tumefaciens* strain EHA105 were grown at 37 °C and 28 °C on Luria–Bertani (LB) agar plates or in LB broth with shaking (200 rpm), respectively. The antibiotics used were ampicillin (100 μg/mL), kanamycin (50 μg/mL), rifampicin (50 μg/mL), spectinomycin (50 μg/mL), tetracycline (10 μg/mL in liquid medium and 3 μg/mL in solid medium) and hygromycin B (20 μg/mL) for *Arabidopsis* transformant selection.

Protein expression, purification and antibody preparation

The *pip* coding sequence from the *Xcc* 8004 genome was PCR amplified with pip *Nde*I F and pip *Xho*I R primers and confirmed as correct by sequencing. The amplicon was ligated to the pET30a vector to generate the protein expression vector. Site-directed mutagenesis of S106A, D246A and H273A active site residues in PIP was conducted on pET30a-*pip* using the fast mutagenesis system (Transgen Biotech, Haidian District, China). The resultant expression vectors were transformed individually into *E. coli* BL21 (DE3) for the expression of His6-tagged proteins. The early logarithmic phase [optical density at 600 nm (OD_{600}) = 0.4] *E. coli* culture was induced overnight with 0.3 mM isopropyl-β-D-thiogalactopyranoside at 16 °C with shaking (160 rpm). The pellet was harvested by centrifugation (5 min, 10 000 ***g***, 4 °C), and then suspended in Ni-NTA binding buffer (300 mM NaCl, 10 mM imidazole, 50 mM NaH_2PO_4, pH 8.0) containing 1 mM phenylmethanesulfonyl fluoride, lysed in a high-pressure homogenizer and centrifuged at 10 000 ***g*** for 20 min. The supernatant was affinity purified using Ni-NTA His•Bind Resin to obtain the His-tagged fusion proteins according to the procedure recommended by the manufacturer (Novagen, Whitehouse Station, NJ, USA). The purified His-tagged PIP protein was used to immunize and boost rabbits to obtain antibodies against PIP, and serum was collected after the fourth booster injection.

An Amicon® Ultra-4 centrifugal filter device (molecular weight cut-off, 10 kDa; Millipore, Whitehouse Station, NJ, USA) was used for protein concentration or buffer exchange. The protein purification was examined by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by Coomassie blue staining.

RNA extraction and quantitative RT-PCR analyses

For the bacterial flagella-associated gene expression assays, *Xcc* and its derivative strains were cultured to the mid-logarithmic phase (OD_{600} = 1.0) in NYG medium and harvested by centrifugation. The cell pellet was resuspended in XOLN medium, the cells were cultured for 6 h at 28 °C and then harvested at 4 °C by centrifugation (10 000 ***g***, 5 min). Total RNA was isolated using TRIzol® reagent (Invitrogen, Waltham, MA, USA).

For the SA signalling pathway-related gene expression assay in plants, leaves (0.1 g) were collected and ground to a powder in liquid nitrogen, and 1 mL of TRIzol was added to isolate total RNA from the samples.

Total RNA (1 μg) from the bacteria or plants was used as a template to synthesize cDNA using a ReverTra Ace® qPCR RT Master Mix with the gDNA remover kit (Toyobo, Osaka, Japan). Quantitative real-time RT-PCR analyses were conducted using iQ SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) in a C1000 Touch Thermal Cycler (Bio-Rad, Hercules, CA, USA) with bacterial 16S rRNA as a reference gene to quantify the transcript expression levels of flagella-associated genes, or the *Actin* 2

reference gene to quantify plant SA-related gene expression. The primer sequences are listed in Table S2.

Bacterial motility assays

XOLN medium soft-agar motility plates (0.3% agar) were used to determine the swimming motility of the bacterial strains. After stabbing the bacterial colonies onto these plates, the diameter of the migration zone was measured after 48 h of incubation at 28 °C. One-way analysis of variance (ANOVA) was used to compare the motility differences between the strains. The data reported are representative of three independent experiments, which all gave similar results.

Determination of intracellular c-di-GMP levels

c-di-GMP concentrations were measured in the *Xcc* strains. Each strain was inoculated from frozen stock into XOLN medium and cultured at 28 °C until the OD_{600} reached 0.2. Intracellular levels of c-di-GMP were determined by HPLC-MS/MS according to a previously described procedure (Waters *et al.*, 2008). Briefly, to extract c-di-GMP, 50 mL of each culture was harvested by centrifugation (5000 ***g***, 10 min). The cell pellets were resuspended in 900 μL of cold extraction solution (40% acetonitrile, 40% methanol, 0.1% formic acid, 19.9% distilled water), vortexed for 30 s, incubated on ice for 15 min and then lysed by non-contact ultrasonication (Bioruptor UCD-200, Diagenode, Seraing (Ougrée), Belgium). The supernatants were extracted three times using extraction solution with centrifugation (16 100 ***g***, 10 min) between each step; thereafter they were pooled and dried by lyophilization. The resulting samples were resuspended in 60 μL of HPLC-grade water and analysed by HPLC-MS/MS on an AB SCIEX QTRAP 4500 system (AB SCIEX, Foster City, CA, USA). Reverse-phase liquid chromatography was performed using a Synergi Hydro-RP 80A LC column (4 μm, 150 mm × 2 mm; Phenomenex, Torrance, CA, USA). MS was conducted using electrospray ionization, with the analyses in negative-ion mode. The amount of c-di-GMP in the samples was estimated using a standard curve generated from pure c-di-GMP (Biolog Life Science Institute, Bremen, Germany). c-di-GMP levels were normalized against the total proteins. To determine protein amounts, 3 mL of each culture were pelleted by centrifugation, and the cells were lysed with 300 μL of phosphate-buffered saline (PBS) using an ultrasonic oscillator. After centrifugation (10 000 ***g***, 20 min), the protein concentrations in each supernatant were measured by the Bradford Protein Assay kit (Bio-Rad, Hercules, CA, USA).

Plant inoculation and non-host HR assay

Plasmid pRF419 (Zhang *et al.*, 2007) (renamed here as pLAFR3-*pip*) was transformed into *Xcc* 8004 to generate *Xcc*/pLAFR3-*pip*. The pip^{S106A} fragment in pET30a was PCR amplified with pip *Bam*H I F and pip *Hin*d III R primers. Its sequence was verified and then cloned into pLAFR3 to obtain pLAFR3-pip^{S106A}, and the resultant plasmid was transformed into *Xcc* 8004.

Xcc strains were cultured to OD_{600} = 1.0, the cell numbers were adjusted to 10^8 in 10 mM $MgCl_2$ buffer and then used to inoculate 4-week-old SR1 tobacco or cabbage leaves. The leaves were inspected for HR lesion at 2 days after inoculation.

The PIP and PIP^{S106A} proteins purified from *E. coli* cells were diluted to different concentrations (1, 5 and 10 mg/mL in PBS, using PBS as a control), injected with a needless syringe into tobacco SR1 and cabbage leaves for disease or HR lesion development, and then photographed at 48 h after protein infiltration.

Generation of bacterial in-frame deletion mutants

Xcc 8004 was used as the parental strain for deletion mutant generation. In-frame deletion of *hrcV* was conducted using the primers listed in Table S2 according to previously described methods (Slater *et al.*, 2000). Briefly, two flanking *hrcV* fragments were PCR amplified with two primer pairs (LhrcV F-*Eco*R I/LhrcV R-*Hin*d III and RhrcV F-*Hin*d III/RhrcV R-*Bam*H I). Both fragments were cleaved with *Hin*d III, and then ligated to generate an intermediate fused fragment. The PCR-amplified DNA fusion fragment from LhrcV primer F-*Eco*R I and RhrcV primer R-*Bam*H I primers was cloned into a pK18mobsacB vector. The recombinant plasmid was transformed into *Xcc* 8004 by electroporation. Transformants were selected on LB medium supplemented with rifampicin and kanamycin. Positive colonies were plated onto NYG medium containing 5% (w/v) sucrose and rifampicin to select for the second cross-over event resulting in the loss of the *sacB* gene. The *hrcV* in-frame deletion mutant was verified by sequencing.

Bacterial growth *in planta*

Xcc was resuspended in 10 mM $MgCl_2$ and inoculated at a cell density of 1×10^5 CFU/mL into the leaves of 4-week-old *Arabidopsis* plants with a needleless syringe. The bacterial population in each leaf was counted at 72 hpi using a Petri dish colony counting method. Each data point consisted of at least four replicates.

Adenylate cyclase assays

The first 100-amino-acid coding sequence fragment of PIP was amplified with the primers $pip_{(1–100)}$-Cya *Sac*I F and $pip_{(1–100)}$-Cya *Bam*H I R, verified by sequencing and ligated to pCPP3214 with the Cya coding sequence (Schechter *et al.*, 2004). Next, the $pip_{(1–100)}$-*cya* fragment was digested with *Eco*R I and *Hin*d III, and cloned into pLAFR3 to generate pLAFR3-$pip_{(1–100)}$-*cya*. PCR-amplified DNA fragments coding for the N-terminal 98 amino acids of AvrBs2 (Casper-Lindley *et al.*, 2002) and the full length of enhanced green fluorescent protein (eGFP) (Genbank accession No. KM019171), the obtained fragments were ligated with the large fragment of *Bam*H I/*Eco*R I-digested pLAFR3-$pip_{(1–100)}$-*cya* by a seamless assembly cloning kit (Trelief SoSoo Cloning Kit Ver. 2, TSINGKE, Changping District, China) to generate pLAFR3-AvrBs2$_{(1–98)}$-*cya* and pLAFR3-eGFP$_{(1–244)}$-*cya*. The plasmids were transformed into *Xcc* 8004 and Δ*hrcV* to generate *Xcc* 8004/pLAFR3-$pip_{(1–100)}$-*cya*, Δ*hrcV*/pLAFR3-$pip_{(1–100)}$-*cya*, *Xcc* 8004/pLAFR3-AvrBS2$_{(1–98)}$-*cya* and *Xcc* 8004/pLAFR3-eGFP$_{(1–244)}$-*cya*, respectively.

The bacterial cells and the culture supernatants were collected separately. The supernatants were added to an equal volume of 20% trichloroacetic acid (TCA) and incubated for 30 min on ice for protein precipitation; after centrifugation, the pellets were washed in cold acetone and then dissolved in PBS for western blot. The remaining cells were boiled in SDS lysis buffer containing 50 mM Tris-HCl (pH 6.8), 2% (w/v) SDS, 10% (v/v) glycerol, 1% (v/v) β-mercaptoethanol (2-ME) and 0.1% (w/v) BPB

(bromphenol blue). Protein samples were separated by electrophoresis on 10% SDS-PAGE gels and transferred to PVDF (polyvinylidene fluoride) (Millipore, Whitehouse Station, NJ, USA) membranes using a semidry transfer system (Bio-Rad, Hercules, CA, USA). Western blot working solution was prepared by mixing 100 μL of the stable peroxide solution and the luminol/enhancer solution (Promega, Madison, WI, USA). Cya fusion proteins were detected using primary anti-Cya (3D1) mouse monoclonal IgG antibody (Santa Cruz Biotechnology, Dallas, TX, USA).

Adenylate cyclase activity assays in plant tissue were conducted as described previously (Schechter *et al.*, 2004). Briefly, 0.1–0.2 g of leaves were collected at 10 hpi by *Xcc* strains (OD_{600} = 0.8), frozen in liquid nitrogen, ground to a powder and suspended in 100 μL of 0.1 M HCl. The concentrations of cAMP were measured in leaf samples using a cAMP complete enzyme-linked immunosorbent assay (ELISA) kit (Enzo Life Sciences, Farmingdale, NY, USA) according to the manufacturer's instructions. The protein content of each sample was determined by the Bio-Rad protein assay (Bio-Rad, Hercules, CA, USA) for cAMP level normalization. The mean values obtained represent data triplicates from each separate experiment.

PIP enzymatic activity assays

PIP activity was assayed with L-proline *p*-nitroanilide trifluoroacetate salt (PPNA) as a substrate as described previously (Zhang *et al.*, 2007). The reaction was performed according to the procedure of Sigma (http://www.sigmaaldrich.com/content/dam/sigma-aldrich/docs/Sigma/General_Information/proline_iminopeptidase.pdf) at 37 °C for 10 min. The purified PIP, PIP^{S106A}, PIP^{D246A} and PIP^{H273A} proteins were extracted from *E. coli* cells using PBS; 5 μg of protein of each sample was used to assay the enzyme activity.

Transgenic *Arabidopsis* leaves (about 0.1 g) were frozen with liquid nitrogen and ground to a powder. PIP or PIP^{S106A} protein was extracted using 500 μL of NP-40 buffer (50 mM Tris-HCl, 100 mM KCl, 2.5 mM $MgCl_2$, 0.1% NP-40) and centrifuged at 10 000 ***g*** for 30 min; the total protein in the supernatant was quantified by the Bio-Rad protein assay system (Bio-Rad, Hercules, CA, USA). For PIP enzymatic assay, 100 μg of total protein (or 5 μg of PIP protein from *E. coli*) were added to the reaction buffer [80 mM Tris-HCl, pH 8.0, 4 mM dithiothreitol (DTT), 1.5 mM ethylenediaminetetraacetic acid (EDTA) and 0.17 mM PPNA] and incubated at 37 °C for 10 min, using sodium acetate (pH 4.0) (final concentration, 250 mM) to stop the reaction. The absorbance of *p*-nitroaniline released by PIP was measured at 410 nm.

The relative enzymatic activities of PIP variant proteins were calculated with respect to the established mean of wild-type PIP activity. Four independent experiments were performed in triplicate.

Construction of transgenic plants

To create DNA encoding N-terminal 3×FLAG-PIP (or 3×FLAG-PIP^{S106A}) fusion proteins, PCR was conducted with the primers pip N-flag *Bam*H I F and pip N-flag *Sac*I R using pET30a-*pip* (or pET30a-pip^{S106A}) as a template. The PCR products were cloned into the pGEM-T vector (Promega, Madison, WI, USA) and verified by DNA sequencing, and the resulting *Bam*H I-*Sac*I fragment was fused to the binary expression vector pCAMBIA1300, followed by transformation into the *Agrobacterium tumefaciens* strain EHA105. *Arabidopsis thaliana* (ecotype Col-0) was transformed using the floral dip method by *Agrobacterium tumefaciens* (containing the transgenes *pip* or pip^{S106A}) strains as described previously (Clough and Bent, 1998; Zhang *et al.*, 2006). Treated plants were allowed to set seeds, which were then plated onto hygromycin B (20 μg/mL) medium for transformant selection. Total protein was extracted from the transgenic plants, and 50 μg was size fractionated on a 10% SDS-PAGE gel. The PIP and PIP^{S106A} expression levels in transgenic plants were verified by western blotting using anti-PIP rabbit polyclonal antibody.

SA measurement using HPLC-MS/MS

Plants were infected with *Xcc* 8004 using a needless syringe. One leaf on each plant was infiltrated with a suspension of *Xcc* (OD_{600} = 0.5). At 12 hpi, free SA measurement was performed with the leaf tissues from 4-week-old plants, as described previously (Bowling *et al.*, 1994). Briefly, 0.1 g of leaves were ground into a powder in liquid nitrogen; 1 mL of 90% methanol was added, the sample was centrifuged at 14 000 ***g*** for 10 min and the supernatant was transferred to a new tube. The pellet was re-extracted with 0.5 mL of 100% methanol, and the supernatant was combined with the first-step supernatant, and dried in a speed vacuum to a final volume of ~50 μL. The residue was resuspended in 500 μL of 0.1 M sodium acetate (pH 5.5), and an equal volume of 10% TCA was added to the tube. After centrifugation at 14 000 ***g*** for 10 min, the supernatant was transferred to a new tube and partitioned with 1 mL of ethylacetate–cyclohexane (1 : 1). After centrifugation at 14 000 ***g*** for 10 min, the organic phase was transferred to a new tube, dried in a speed vacuum, dissolved in 150 μL of 100% methanol and re-centrifuged at 14 000 ***g*** for 10 min. The supernatant was used for HPLC-MS/MS analysis on an AB SCIEX QTRAP 4500 system (AB SCIEX). Reverse-phase liquid chromatography was performed with an Agilent Extend C18 column (3.5 μm, 2.1 mm × 100 mm; Agilent, Santa Clara, CA, USA). The sample was eluted with 0.8% acetic acid (pH 5.5) (solvent A), 100% acetonitrile (solvent B) and A : B (75 : 25) at a flow rate of 0.40 mL/min. The quantity of SA in each sample was estimated using a standard curve generated from pure SA (Sigma, Whitehouse Station, NJ, USA). SA levels were normalized against the fresh leaf weight.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Table S1 Tested flagellar genes in *Xanthomonas campestris* pv. *campestris* (*Xcc*) 8004.

Table S2 Bacterial strains and plasmids used in this work.