BDSF Is the Predominant In-Planta Quorum-Sensing Signal Used During *Xanthomonas campestris* Infection and Pathogenesis in Chinese Cabbage

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Xanthomonas campestris pv. *campestris* uses the diffusible signal factor (DSF) family of quorum-sensing (QS) signals to coordinate virulence and adaptation. DSF family signals have been well-characterized using laboratory-based cell cultures. The in-planta QS signal used during *X. campestris* pv. *campestris* infection remains unclear. To achieve this goal, we first mimic in-planta *X. campestris* pv. *campestris* growth conditions by supplementing the previously developed YYS medium with cabbage hydrolysate and found that the dominant signal produced in these conditions was BDSF. Secondly, by using YYS medium supplemented with diverse plant-derived compounds, we examined the effects of diverse plant-derived compounds on the biosynthesis of DSF family signals. Several compounds were found to promote biosynthesis of BDSF. Finally, using an *X. campestris* pv. *campestris* Δ rpfB–Chinese cabbage infection model and an ultra-performance liquid chromatographic-time of flight-mass spectrometry–based assay, BDSF was found to comprise >70% of the DSF family signals present in infected cabbage tissue. BDSF at a concentration of 2.0 μ M induced both protease activity and *engXCA* expression. This is the first report to directly show that BDSF is the predominant in-planta QS signal used during *X. campestris* pv. *campestris* infection. It provides a better understanding of the molecular interactions between *X. campestris* pv. *campestris* and its cruciferous hosts and also provides the logical target for designing strategies to counteract BDSF signaling and, thus, infection. Further studies are needed to get an exact idea about the DSF production dynamics of the wild-type strain inside the plant.

Bacteria are able to sense and respond to changes in their populations by communicating with small signal molecules, a

mechanism known as quorum sensing (QS). The diffusible signal factor (DSF) family represents a new type of QS signal molecule that is found in diverse gram-negative bacteria, including *Xanthomonas campestris*, *Xylella fastidiosa*, *Burkholderia cepacia*, and *Pseudomonas aeruginosa* (Deng et al. 2011; He and Zhang 2008). DSF family signals are *cis*-2-unsaturated fatty acids, containing fatty acid carbon chains with variations in length, double-bond configuration, and side-chain composition (Zhou et al. 2017). DSF family signals have been shown to regulate motility, biofilm formation, iron uptake, exopolysaccharide (EPS) and extracellular enzyme production, and virulence in these bacterial pathogens (Deng et al. 2011; He and Zhang 2008). Recently, the DSF family signals were found to provide a fitness advantage in mixed culture (Dean et al. 2015; Deng et al. 2016; Han et al. 2015). Pathogen-derived DSF signal elicits host plant innate immunity (Kakkar et al. 2015). These new findings demonstrate that DSF family signals are not only important for communication within a homogeneous bacterial population but, also, function in the areas of interspecies and interkingdom communication (Zhou et al. 2017).

The crucifer pathogen *Xanthomonas campestris* pv. *campestris* is one of the model bacteria used in studying DSF family signaling. *X. campestris* pv. *campestris* contains a *rpf* gene cluster that encodes the key DSF signaling components RpfB, RpfF, RpfC, and RpfG (Slater et al. 2000; Tang et al. 1991). RpfF is a key enzyme required for DSF biosynthesis, whereas RpfC and RpfG constitute a two-component regulatory system involved in DSF signal perception and transduction (Cai et al. 2017; He et al. 2006a; Slater et al. 2000). The activated RpfG has phosphodiesterase activity and is able to degrade cyclic di-GMP (c-di-GMP). C-di-GMP is an inhibitory ligand of the transcription factor Clp (Tao et al. 2010). The de-repressed Clp further regulates the expression of several hundreds of genes, including those virulence factor genes (He et al. 2007; Tao et al. 2010). RpfB has fatty acyl-CoA ligase activity and is involved in the turnover of the DSF family signals in *X. campestris* pv. *campestris* (Wang et al. 2016; Zhou et al. 2015).

In *X. campestris* pv. *campestris* DSF biosynthesis is negatively controlled by RpfC via a mechanism involving RpfC-RpfF interactions (He and Zhang 2008; He et al. 2006a). RpfF has both dehydratase and thioesterase activities and uses 3-hydroxyacyl-ACP as a direct substrate to synthesize DSF family signals. The substrate 3-hydroxyacyl-ACP is further derived from the classic fatty acid synthesis pathway (Hu et al. 2018; Zhou et al. 2015). Interestingly, several bacterial species produce one or more DSF family signals, such as DSF

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(*cis*-11-methyl-2-dodecenoic acid), BDSF (*cis*-2-dodecenoic acid), CDSF (*cis*-11-methyldodeca-2,5-dienoic acid), IDSF (*cis*-10-methyl-2-dodecenoic acid), *cis*-9-methyl-2-decenoic acid, and *cis*-2-undecenoic acid (Deng et al. 2010; He et al. 2010; Zhou et al. 2015; Deng et al. 2016). The relative proportions of these signals are influenced by the composition of the culture medium in which the bacteria are grown. Zhou et al. (2015) revealed that *X. campestris* pv. *campestris* produces mainly BDSF in the media containing different carbohydrates as carbon sources. Methyl substitutions in DSF originate from leucine and valine and from isoleucine in IDSF (Zhou et al. 2015). RpfF could use multiple types of acyl-ACP precursors for the synthesis of DSF family signals (Zhou et al. 2015).

X. campestris pv. *campestris* is a vascular pathogen and normally gains entry to the plant via leaf margin hydathodes, stomata, or wounds (Hugouvieux et al. 1998). Once inside the plant, *X. campestris* pv. *campestris* uses plant-derived nutrients to multiply and form microcolonies, while producing a range of extracellular enzymes that degrade plant cells. *X. campestris* pv. *campestris* then disperses and colonizes the host plant vascular system, in which it produces extracellular enzymes and the extracellular polysaccharide called EPS or xanthan gum (Onsando 1992; Williams 1980). During all stages of disease development, QS mechanisms are important for the production of extracellular enzymes and other virulence factors (He and Zhang 2008). However, all studies on *Xanthomonas* QS to date have been conducted in laboratory-based cell cultures and very little is known about the QS process during host plant infection. This study is aimed to determine the in-planta QS signals used by *Xanthomonas* spp. during the infection process.

RESULTS

The strains for investigating in-planta QS signals in this study.

During growth in culture, *X. campestris* pv. *campestris* wild-type levels of DSF and BDSF are extremely low, making them very difficult to identify and characterize (Wang et al. 2004). This is because the synthase RpfF is repressed by RpfC (Cheng et al. 2010; He et al. 2006a) and, also, because during late growth stages, RpfB is induced and degrades DSF signals (Zhou et al. 2015). To overcome these problems, the *rpfC* and *rpfB* double-deletion strain $\Delta rpfC\Delta rpfB$, which constitutively produces DSF and BDSF at high levels (Zhou et al. 2015), was used to conveniently and accurately monitor the DSF family signals produced in culture. The use of this strain was further supported by our previous finding that RpfB has the same activity against DSF and BDSF in vivo. The ratios of DSF and BDSF in *rpfB* deletion or overexpression strains were similar to that of the wild-type strain (Wang et al. 2016; Zhou et al. 2015). Moreover, since the $\Delta rpfB$ strain was previously shown to be more virulent in Chinese radish and to produce higher levels of DSF and BDSF than the wild-type strain (Zhou et al. 2015), it greatly facilitated the establishment of the *X. campestris* pv. *campestris*-cabbage infection and the detection of DSF family signals in the infected cabbage tissue.

Biosynthesis of DSF family signals in the presence of plant hydrolysate.

The nutrients available for *X. campestris* pv. *campestris* growth and development in planta are not likely to be solitary but, instead, a mixture of multiple components. To mimic these in-planta growth conditions, hydrolysate was extracted from Chinese cabbage, using previously described methods (Xu et al. 2010). The cabbage hydrolysate was then added to $\Delta rpfC\Delta rpfB$ XY (XOLN salts with 0.02% yeast extract) or YYS (XY with 0.2% sucrose) medium culture at a final concentration of either 1

or 2%. The levels of DSF and BDSF in the cultures were determined and were then expressed as concentrations of micromoles per 10^9 cells, following previously described methods (Zhou et al. 2015). In XY medium, strain $\Delta rpfC\Delta rpfB$ produced both BDSF (0.06×10^{-3} $\mu\text{mol}/10^9\text{cells}$) and DSF (0.11×10^{-3} $\mu\text{mol}/10^9\text{cells}$) (Fig. 1B). The addition of 1 or 2% plant hydrolysate to medium XY increased bacterial growth from 20.0 to 40% (Fig. 1A). In the presence of plant hydrolysate, the BDSF levels of the $\Delta rpfC\Delta rpfB$ cultures were 0.26×10^{-3} $\mu\text{mol}/10^9\text{cells}$ (1% hydrolysate) and 0.38×10^{-3} $\mu\text{mol}/10^9\text{cells}$ (2% hydrolysate), which are 3.3- and 5.3-fold increases over that of XY culture without hydrolysate, respectively (Fig. 1B). The DSF levels in the presence of cabbage hydrolysate were 0.15×10^{-3} $\mu\text{mol}/10^9\text{cells}$ (1% hydrolysate) and 0.22×10^{-3} $\mu\text{mol}/10^9\text{cells}$ (2% hydrolysate), which are 0.4- and 1.0-fold increases over that of the XY culture without hydrolysate, respectively (Fig. 1B).

In YYS medium, strain $\Delta rpfC\Delta rpfB$ produces more BDSF (0.47×10^{-3} $\mu\text{mol}/10^9\text{cells}$) than DSF (0.07×10^{-3} $\mu\text{mol}/10^9\text{cells}$) (Fig. 1D). In this medium, the addition of plant hydrolysate to the $\Delta rpfC\Delta rpfB$ culture had little effect on bacterial growth (Fig. 1C); however, BDSF levels were significantly increased. BDSF levels of $\Delta rpfC\Delta rpfB$ cultures with the addition of cabbage hydrolysate reached 1.0×10^{-3} $\mu\text{mol}/10^9\text{cells}$ (1% hydrolysate) and 1.6×10^{-3} $\mu\text{mol}/10^9\text{cells}$ (2% hydrolysate), increases of 1.1 and 2.4-fold, respectively. In contrast, DSF levels observed in YYS cultures supplemented with cabbage hydrolysate were still quite low, at 0.11×10^{-3} $\mu\text{mol}/10^9\text{cells}$ (1% hydrolysate) and 0.18×10^{-3} $\mu\text{mol}/10^9\text{cells}$ (2% hydrolysate) (Fig. 1D).

Deng et al. (2015) conducted similar assay in *X. campestris* pv. *campestris* and found that addition of crucifer plant juice or ethanol extract to *X. campestris* pv. *campestris* growth media significantly increased DSF family signal production. No dominant QS signal was reported. To further explore the potential mechanisms underlying the roles of a range of plant-derived compounds on the biosynthesis of DSF family, signals were investigated in the following sections.

The effects of non-branched chain amino acids on the biosynthesis of DSF family signals.

Plants are a rich source of amino acids that perform various prominent roles and functions for the plant (Hildebrandt et al. 2015) and also provide important nutrition for *X. campestris* pv. *campestris* growth in planta. Our previous results showed that the addition of the branched-chain amino acids valine and leucine or isoleucine to *X. campestris* pv. *campestris* $\Delta rpfC\Delta rpfB$ in YYS medium promoted DSF or IDSF biosynthesis, respectively (Zhou et al. 2015). In this study, we investigated the effects of 15 non-branched chain amino acids on the biosynthesis of the DSF family. Exogenous addition of these non-branched chain amino acids to YYS medium at a final concentration of 0.1 or 0.5 mM had little effect on bacterial growth. The $\Delta rpfC\Delta rpfB$ strain grew to a maximum optical density at 600 nm (OD_{600}) of 1.2 at 24 h after inoculation, regardless of amino acid additions to culture medium (Supplementary Figs. S1, S2, S3, and S4). Four amino acids, cysteine, glutamate, phenylalanine, and histidine, at concentrations of either 0.1 or 0.5 mM, significantly increased BDSF production (Fig. 2), and glutamate significantly increased DSF levels (Fig. 3). The effect of glutamate on DSF biosynthesis was less significant than that of leucine and valine (Fig. 3). In contrast, addition of the 15 non-branched chain amino acids to YYS medium had little effect on IDSF biosynthesis (Supplementary Fig. S5).

To further investigate how these non-branched chain amino acids affect BDSF or DSF biosynthesis, the expression level of RpfF, a key enzyme involved in DSF biosynthesis, was investigated in the presence of the above-mentioned four amino

Fig. 1. Effects of plant hydrolysate on diffusible signal factor (DSF) and BDSF biosynthesis. **A**, Growth curve of *Xanthomonas campestris* pv. *campestris* in XY (XOLN salts with 0.02% yeast extract) medium. **B**, DSF and BDSF production of the strain $\Delta rpfC\Delta rpfB$ in XY medium supplemented with cabbage hydrolysate at concentrations of either 1 or 2%. **C**, Growth curve of *X. campestris* pv. *campestris* in YYS (XY with 0.2% sucrose) medium. **D**, DSF and BDSF level of the strain $\Delta rpfC\Delta rpfB$ observed in YYS medium supplemented with cabbage hydrolysate at concentrations of either 1 or 2%. Each experiment was repeated twice, the data shown are the average of three replicates and error bars represent standard deviations. Statistically significant differences with respect to the non-amended XY medium are indicated by one asterisk ($P \leq 0.05$).

acids. Western blotting analysis showed that none of these amino acids had a significant effect on RpfF levels, suggesting that these four amino acids probably promote BDSF biosynthesis in another manner, such as through metabolic processes.

The effects of branched-chain α -ketoacids and carboxylic acids on the biosynthesis of DSF family signals.

In bacteria, the branched-chain fatty acids are synthesized from branched-chain acyl-CoA with malonyl-CoA as the chain extender (Kaneda 1991). The branched-chain acyl-CoA can be synthesized from the branched-chain α -ketoacids, including α -ketoisocaproic acid (KIC), α -ketoisovaleric acid (KIV), and α -keto- β -methylvaleric acid (KMV). These α -ketoacids are derived from the catabolism of the branched-chain amino acids, i.e., leucine, valine, and isoleucine (Beck 2005). Alternatively, the exogenously supplied short-chain carboxylic acids, including isobutyric acid (IBA), isovaleric acid (IVA), and 2-methylbutyric acid (2MBA), can be used for branched-chain acyl-CoA biosynthesis (Ward et al. 1999). To investigate whether these α -ketoacids and carboxylic acids influence the biosynthesis of DSF family signals, they were separately added to *X. campestris* pv. *campestris* $\Delta rpfC\Delta rpfB$ cultures at a final concentration of 0.01, 0.1, or 0.5 mM. Although the exogenous addition of three α -ketoacids had no significant effects on the growth of strain $\Delta rpfC\Delta rpfB$, analysis of the culture supernatants 24 h after inoculation revealed that the addition of either KIC or KIV increased DSF levels whereas the addition of KMV increased IDSF levels (Fig. 4).

Although exogenous addition of 0.1 mM carboxylic acid to the $\Delta rpfC\Delta rpfB$ culture had no significant effect on bacterial growth, 0.5 mM carboxylic acid did significantly inhibit *X. campestris* pv. *campestris* growth. Regardless, analysis of the culture supernatants at 24 h after inoculation revealed that the three carboxylic acids had little effect on the biosynthesis of DSF family signals (Supplementary Figs. S6).

The effects of plant-derived organic acids on the biosynthesis of DSF family signals.

Shikimic acid, citric acid, malic acid, and succinic acid are organic acids that are important in the basic metabolism of all organisms. To determine the potential effects of these organic acids on the biosynthesis of DSF family signals, $\Delta rpfC\Delta rpfB$ cultures were supplemented with each of the four organic acids, separately, at final concentrations of 0.01, 0.1, or 0.5 mM. Analysis of the results revealed that these organic acids had no significant effect on the biosynthesis of DSF family signals (Supplementary Fig. S7).

The effects of plant hormones on the biosynthesis of DSF family signals.

Plant hormones are signal molecules produced within the plant to regulate growth and development. In order to understand their effects on the biosynthesis of DSF family signals, abscisic acid (ABA), gibberellic acid (GA3), indol-3-acetic acid (IAA), epibrassinolide (EBL), jasmonate (JAS), and salicylic acid (SA) were separately added to $\Delta rpfC\Delta rpfB$ cultures at final concentrations of either 0.01 or 0.10 mM. Although these phytohormones had little effect on bacterial growth (Fig. 5A), analysis of the $\Delta rpfC\Delta rpfB$ cultures 24 h after inoculation showed that the addition of ABA and GA3 significantly increased DSF levels and decreased BDSF levels (Fig. 5B and C). In contrast, the addition of SA significantly increased BDSF levels and decreased DSF levels (Fig. 5B and C). The other plant hormones had little effect on DSF and BDSF biosynthesis. Furthermore, Western blotting analysis showed that the addition of GA3 and SA had little effect on RpfF levels, but the addition of ABA at a concentration of 100 μ M significantly decreased RpfF levels (Fig. 5D).

The effects of plant-derived phenolic compounds on the biosynthesis of DSF family signals.

Naturally occurring phenolic compounds are found at high levels in plants and play a major role in plant reproduction and growth as well as defense against pathogens (Báidez et al. 2007; Huang et al. 2010). To determine the possible role of

phenolic compounds in DSF family signal biosynthesis, strain $\Delta rpfC\Delta rpfB$ was grown for 24 h in YYS medium supplemented with 3-hydroxybenzoic acid (3-HBA), 4-HBA, vallinic acid, gallic acid, trans-2 hydroxycinnamic acid (2-HCA), 3,4-dihydroxybenzoic acid, ferulic acid, or caffeic acid at final concentrations of 0.1 or 0.5 mM. The addition of these

Fig. 2. Diffusible signal factor BDSF biosynthesis by *Xanthomonas campestris* pv. *campestris* $\Delta rpfB\Delta rpfC$ in the presence of different amino acids. Strain $\Delta rpfC\Delta rpfB$ was grown in YYS (XOLN salts with 0.02% yeast extract with 0.2% sucrose) medium containing 0.1 or 0.5 mM amino acids at 30°C for 24 h. Each bar represents the average of three independent experiments and error bars represent standard deviations. Statistically significant differences with respect to the nonamended YYS medium are indicated by one or two asterisks ($P \leq 0.05$ or 0.01, respectively).

compounds to XYS culture medium had little effect on bacterial growth (Fig. 6A). However, the addition of either 4-HBA or 2-HCA significantly increased BDSF levels in the cultures (Fig. 6B and C), and 3-HBA at a concentration of 0.5 mM significantly decreased DSF levels. None of the other phenolic

acids had significant effects on the biosynthesis of DSF family signals (Fig. 6B and C). Furthermore, Western blotting analysis showed that the addition of 2-HCA significantly increased levels of RpfF, although the addition of 4-HBA had little effect on RpfF levels (Fig. 6D).

Fig. 3. Diffusible signal factor (DSF) biosynthesis by strain $\Delta rpfB\Delta rpfC$ in the presence of different amino acids. The $\Delta rpfC\Delta rpfB$ strain was grown in XYS (XOLN salts with 0.02% yeast extract with 0.2% sucrose) media containing 0.1 or 0.5 mM amino acids at 30°C for 24 h. Each bar represents the average of three independent experiments and error bars represent standard deviations. Statistically significant differences with respect to the nonamended XYS medium are indicated by one or two asterisks ($P \leq 0.05$ or 0.01 , respectively).

DSF family signals

in *X. campestris* pv. *campestris*-infected cabbage.

To further understand the QS signals utilized by *X. campestris* pv. *campestris* in planta during the infection process, we used an *X. campestris* pv. *campestris* $\Delta rpfB$ strain to

infect Chinese cabbage, because the *rpfB* mutant strain is more virulent on Chinese radish and produces higher levels of DSF family signals than the wild-type strain (Zhou et al. 2015). At different stages of infection, the infected cabbages were collected to monitor bacterial growth by counting the

Fig. 4. Diffusible signal factor (DSF) family signal biosynthesis by strain $\Delta rpfB\Delta rpfC$ in the presence of different α -ketoacids. **A**, BDSF biosynthesis. **B**, DSF biosynthesis. **C**, IDSF biosynthesis. *X. campestris* pv. *campestris* $\Delta rpfB\Delta rpfC$ was grown in YYS (XOLN salts with 0.02% yeast extract with 0.2% sucrose) medium containing α -keto acids at a concentration of either 0.1 or 0.5 mM at 30°C for 24 h. Each bar represents the average of three independent experiments and error bars represent standard deviations. Statistically significant differences with respect to the nonamended YYS medium are indicated by one or two asterisks ($P \leq 0.05$ or 0.01, respectively). KMV = α -keto- β -methylvaleric acid, KIV = α -ketoisovaleric acid, and KIC = α -ketoisocaproic acid.

colony number per gram of fresh weight and to extract DSF family signals. Methods for extracting and purifying DSF family signals from infected cabbage plants were established, and the quantitative analysis of DSF family signals

was optimized based on latest description by Zhou et al. (2018).

Strain $\Delta rpfB$ effectively established infections in Chinese cabbage plants and black rot disease was observed

Fig. 5. Diffusible signal factor (DSF) family signal biosynthesis by strain $\Delta rpfB\Delta rpfC$ in the presence of different plant hormones. **A**, Bacterial growth curves of *X. campestris* pv. *campestris* strains. **B**, BDSF biosynthesis. **C**, DSF biosynthesis. **D**, Western blotting analysis showing the effects of abscisic acid (ABA), gibberellic acid (GA3), and salicylic acid (SA) on RpfF expression. *Xanthomonas campestris* pv. *campestris* $\Delta rpfB\Delta rpfC$ was grown in YYS (XOLN salts with 0.02% yeast extract with 0.2% sucrose) medium containing plant hormones at a concentration of either 0.01 or 0.1 mM at 30°C for 24 h. Each bar represents an average of three independent experiments and error bars represent standard deviations. Statistically significant differences with respect to the nonamended YYS medium are indicated by one or two asterisks ($P \leq 0.05$ or 0.01 , respectively). IAA = indol-3-acetic acid, EBL = epibrassinolide, and JAS = jasmonate. The numbers indicate the Western blotting signal intensities of RpfF as measured using ImageJ software.

2 weeks after inoculation (Fig. 7A). The average number of *X. campestris* pv. *campestris* cells inside the infected cabbage were 17.1 per gram of fresh weight (FW) (first week), 65.4 per gram of FW (second week), and 86.5 per gram of FW (third

week), respectively (Fig. 7C). No DSF or BDSF was detectable in the infected tissues 1 week after inoculation (Fig. 7C). The levels of DSF and BDSF at 2 to 3 weeks after inoculation reached 0.065 and 0.142 pmol per gram of FW, respectively

Fig. 6. Diffusible signal factor (DSF) family signal biosynthesis by strain $\Delta rpfB\Delta rpfC$ in the presence of different plant-derived phenolics. **A**, Growth curves of *X. campestris* pv. *campestris* strains. **B**, BDSF biosynthesis. **C**, DSF biosynthesis. *X. campestris* pv. *campestris* $\Delta rpfC\Delta rpfB$ was grown in XYZ (XOLN salts with 0.02% yeast extract with 0.2% sucrose) medium containing plant-derived phenolics at concentrations of either 0.1 or 0.5 mM at 30°C for 24 h. Each bar represents the average of three independent experiments and error bars represent standard deviations. Statistically significant differences with respect to the nonamended XYZ medium are indicated by one asterisk ($P \leq 0.05$). 3-HBA = 3-hydroxybenzoic acid, 4-HBA = 4-hydroxybenzoic acid, VAA = vallinic acid, GAA = gallic acid, 2-HCA = *trans*-2 hydroxycinnamic acid, DHBA = 3,4-dihydroxybenzoic acid, FEA = ferulic acid, and CAA = caffeic acid. The numbers indicate the Western blotting signal intensities of RpfF as measured using ImageJ software.

(Fig. 7C). Two major DSF family signals, BDSF and DSF were present in the infected cabbage tissue 2 weeks after inoculation (Fig. 7B and C). BDSF comprised 73.3% of the DSF family signals, and DSF was 26.7% of the total (Fig. 7C). In the infected cabbage tissue 3 weeks after inoculation, DSF and BDSF comprised 19.3 and 80.7% of the DSF family signals, respectively (Fig. 7C). No DSF family signals were detected from the phosphate buffered saline (PBS)-treated cabbage (data not shown).

BDSF induces endoglucanase gene expression and protease activity in *X. campestris* pv. *campestris*.

To further confirm that BDSF is a functional QS signal controlling virulence gene expression in *X. campestris* pv. *campestris*, it was added to BDSF-deficient *rpjF* mutant cultures at final concentrations ranging from 0.1 to 2.0 μ M. After 24 h of growth, protease activity and *engXCA* expression levels were compared with those of the wild-type strain. Exogenous addition of BDSF at 0.1 or 0.5 μ M had little effect on *engXCA* expression and protease activity (data not shown). However, the addition of BDSF at 1.0 μ M partially restored the protease activity and *engXCA* expression of the *rpjF* mutant to wild-type levels and addition of BDSF at 2.0 μ M fully restored these traits (Fig. 8A and B).

DSF is commonly used in *X. campestris* pv. *campestris* QS studies, since it was the first identified member of the DSF family and is the major QS signal produced in rich medium (Wang et al. 2004; Zhou et al. 2015). In this study, we further compared the inductive activity of BDSF and DSF at 1.0 and 2.0 μ M in the Δ *rpjF* culture. The protease-inducing activity of BDSF at a 1.0 μ M concentration was approximately 55% that of DSF at 1.0 μ M; however, BDSF at a concentration of 2.0 μ M had an effect comparable to that of DSF at 2.0 μ M (Fig. 8C). Furthermore, the addition of BDSF at a concentration of 2.0 μ M to the strain Δ *rpjF* Δ *rpjC* had no effect on protease activity (Fig. 8C), suggesting that BDSF signaling may also be mediated by the receptor RpfC.

DISCUSSION

To successfully infect a plant, a pathogen must evolve the ability to utilize plant nutrients, establish, and disperse, and, at the same time, neutralize the host plant defenses. In order to study the in-planta QS signals synthesized by *X. campestris* pv. *campestris* during host infection, we developed three strategies that involved two *X. campestris* pv. *campestris* strains, Δ *rpjB* and Δ *rpjB* Δ *rpjC*. We first mimic in-planta *X. campestris* pv. *campestris* growth conditions by supplementing the previously developed XY5 or XY medium with cabbage hydrolysate and found that the dominant signal produced in these conditions was BDSF. We then examined the effects of a range of specific plant-derived compounds on the biosynthesis of DSF family QS signals. Finally, we used an *X. campestris* pv. *campestris* Δ *rpjB* strain to infect Chinese cabbage and analyzed the exact composition of DSF family signals produced in infected cabbage.

***X. campestris* pv. *campestris* uses diverse plant-derived compounds to promote BDSF biosynthesis.**

Amino acids are abundant plant constituents and can be found at various concentrations in plants. Glutamic acid and aspartic acid are the most abundant amino acids in plants (Kumar et al. 2017). During the late stages of disease development, *X. campestris* pv. *campestris* is normally restricted to the xylem vessels of infected plants. Xylem fluid contains carbohydrates, inorganic ions, and organic substances such as amino acids (Sato 2006). *X. campestris* pv. *campestris* cells are, therefore, able to use many of these nutrients for growth

and multiplication (Akimoto-Tomiya et al. 2014). Our previous results showed that the addition of carbohydrates to *X. campestris* pv. *campestris* culture medium promotes BDSF biosynthesis, whereas the addition of the branched-chain amino acids leucine and valine or isoleucine leads to DSF or IDSF biosynthesis, respectively (Zhou et al. 2015). The present study showed that the addition of the non-branched chain amino acids glutamate, cysteine, phenylalanine, and histidine significantly increases BDSF levels (Fig. 2). All these amino acids had no effect on RpfF levels, suggesting that they are probably used as carbon sources to be involved in BDSF biosynthesis. Interestingly, these four non-branched chain amino acids are also abundant in the xylem fluid of *Arabidopsis thaliana* (Sato 2006), soybean (Krishnan et al. 2011), wheat and barley (Goatley and Lewis 1966), and *Petasites japonicus* var. *giganteus* and *Polygonum cuspidatum* (Mizuno et al. 2002). However, very few branched-chained amino acids have been detected in the xylem fluids of these plants. Taken together, these findings suggest that *X. campestris* pv. *campestris* might use carbohydrates and non-branched chain amino acids found in the plant xylem as important nutrients to favor the synthesis of BDSF over that of DSF.

We also found that the addition of two α -ketoacids, KIV and KIC, significantly increased DSF levels, and the addition of KIV increased IDSF levels (Fig. 4). This is highly consistent with the fact that these branched-chain α -ketoacids are derived from the catabolism of leucine, valine, and isoleucine (Beck 2005). *X. campestris* pv. *campestris* encodes an α -ketoacid dehydrogenase that converts them to acetyl-CoA primers for DSF and IDSF biosynthesis (Zhou et al. 2015). The short-chain carboxylic acids IBA, IVA, and 2MBA are also related to valine, leucine, and isoleucine (Ward et al. 1999). However, the addition of IBA, IVA, or 2MBA to XY5 medium had little effect on BDSF, DSF, and IDSF levels. This is probably because either there is no suitable enzyme present in *X. campestris* pv. *campestris* to convert these compounds to acetyl-CoA primers or, alternatively, it is possible that *X. campestris* pv. *campestris* does not have a mechanism for the uptake of these carboxylic acids. Although the three α -ketoacids tested do promote DSF and IDSF biosynthesis in cell culture, their endogenous levels in plants are extremely low; thus, their contribution to in-planta QS signal biosynthesis remains to be elucidated.

Plant hormones are chemicals that regulate essential plant processes such as seed development, flowering time and sex determination, leaf senescence, fruiting, and even plant death. Latest findings suggest that plant hormones not only steer plant immune responses but, also, may influence the virulence of the pathogen. For example, exogenous addition of SA to the opportunistic human pathogen *Pseudomonas aeruginosa* culture significantly reduced the production of motility, biofilm formation, and QS signal (Chow et al. 2011). Recently with the rice bacterial pathogen *X. oryzae* pv. *oryzae*, sodium salicylate (NaSA) induced a nearly 50% increase in DSF levels (Xu et al. 2015). In this study, ABA and GA3 reduced BDSF levels and increased DSF levels (Fig. 5), while SA at a concentration of 0.1 mM increased BDSF levels and decreased DSF levels (Fig. 5). SA and GA3 had little effect on *rpjF* expression, while ABA significantly decreased RpfF levels. These findings suggest that *X. campestris* pv. *campestris* might use the plant hormones SA, ABA, or GA3 as triggers to activate its virulence-associated QS signal biosynthesis. Since ABA and GA3 usually occur at extremely low concentrations in plants (Grennan 2006; Ren et al. 2007), their contributions to in-planta DSF family signal biosynthesis need to be further explored. SA is a phenolic phytohormone that occurs in plants as free SA, carboxylated esters, or phenolic glycosides. It is induced to relatively high levels

upon pathogen invasion (Vlot et al. 2009). Our previous results showed that *X. campestris* pv. *campestris* cannot degrade SA in culture (Wang et al. 2015). The mechanism behind our observed effect of SA on the promotion of BDSF biosynthesis deserves further investigation.

Plants contain significant levels of aromatic metabolites. Many of them play essential roles in plant reproduction and growth as well as in defense against pathogens (Báidez et al. 2007). In response to pathogen attack, plants of the *Brassica*-*ceae* family synthesize, de novo, a broad array of wide

Fig. 7. Identification of diffusible signal factor (DSF) family signals in infected Chinese cabbage. **A**, *Xanthomonas campestris* pv. *campestris* Δ rpfB-infected Chinese cabbage (right); left, Chinese cabbage inoculated with 1× phosphate buffered saline. **B**, Ultra-performance liquid chromatographic-time of flight-mass spectrometry analysis of the DSF family signals extracted from infected cabbage. **C**, The *X. campestris* pv. *campestris* colony number, total level of DSF family signals and ratios of DSF and BDSF in the infected tissues. Statistically significant differences with respect to data 1 week after inoculation are indicated by one or two asterisks ($P \leq 0.05$ or 0.01 , respectively).

spectrum antimicrobial phenolics that accumulate rapidly at areas of pathogen infection (Abdel-Farid et al. 2007; Tan et al. 2004). *X. campestris* pv. *campestris* may also be exposed to high levels of natural phenolic compounds derived from cell-wall degradation during infection. The degradative enzymes produced by *X. campestris* pv. *campestris* not only cleave the cell wall into simple sugars, but they also release lignin to form

various types of aromatic compounds (Bertani et al. 2001). The presence of these phenolic metabolites may affect the biosynthesis of in-planta QS signals during *X. campestris* pv. *campestris* infection. In this study, we observed that the addition of 4-HBA or 2-HCA to *X. campestris* pv. *campestris* cultures significantly increased BDSF levels, and the addition of 3-HBA significantly decreased DSF levels (Fig. 6). In

Fig. 8. Diffusible signal factor BDSF is active in the induction of protease activity and endoglucanase gene expression. **A**, The effects of BDSF on protease activity of the *rpfF* mutant. **B**, The effects of BDSF on the expression of *engXCA*. **C**, A comparison of the inductive effects of diffusible signal factor (DSF) and BDSF at concentrations of either 1.0 or 2.0 μM on protease activity. Data are shown as means $\pm$ one standard deviation of three independent assays.

X. campestris pv. *campestris*, 3-HBA and 4-HBA were previously shown to be involved in the yellow pigment xanthomonadin and coenzyme Q8 biosynthesis, respectively (He et al. 2011; Zhou et al. 2013). DSF family signals, CoQ8, and xanthomonadin all compete for the same primer intermediate acyl-ACP in their biosynthetic pathways (Zhou et al. 2015). The addition of 3-HBA promotes xanthomonadin biosynthesis, resulting in less acyl-ACP available for DSF or BDSF biosynthesis. This is probably why high concentrations of 3-HBA repressed DSF biosynthesis. *X. campestris* pv. *campestris* contains a *pca* gene cluster for the efficient degradation of 4-HBA into PCA, which is further converted into acetyl CoA through the tricarboxylic acid cycle (Wang et al. 2015). The resulting acetyl CoA could be used as a building block for BDSF biosynthesis (Zhou et al. 2015), and this is probably why high concentrations of 4-HBA induced BDSF biosynthesis (Fig. 6). The present study showed that 2-HCA promotes BDSF biosynthesis through increasing RpfF protein levels (Fig. 6). 2-HCA is a derivative of cinnamic acid, which is obtained from the oil of cinnamon, and hydroxycinnamic acid derivatives reportedly have antioxidant activity (Taubert et al. 2003). In addition, some phenolic compounds apparently act as signaling molecules in plant-microbe interactions. For instance, phenolic compounds strongly regulate *nod* gene expression and modify the legume-rhizobial symbiosis in *Rhizobium* spp. (Schultze and Kondorosi 1998). Thus, the mechanism by which 2-HCA regulates *rpfF* expression deserves further investigation.

BDSF is a major in-plant QS signal during infection.

All the DSF family signals characterized from *X. campestris* pv. *campestris* to date were derived from in-vitro cell cultures. Evidence from the present study suggests that BDSF should be the predominant in-plant QS signal in *X. campestris* pv. *campestris* infection and pathogenesis in host plants. Deng et al. (2015) found that the addition of crucifer plant extract significantly increased DSF family signal production in *X. campestris* pv. *campestris*, and the yield of DSF was substantially higher than that of BDSF or DSF-II. The discrepancy between both studies is probably due to the different media used. The results by Deng et al. (2015) were obtained in rich medium. As we have shown that DSF levels are much higher than BDSF levels when *X. campestris* pv. *campestris* was grown in rich nutrient agar and Luria Bertani medium, because these media contain high levels of branched amino acids and related carboxylic acids (Zhou et al. 2015). The media XY or YYS used in this study are relatively poor and close to the growth conditions that *X. campestris* pv. *campestris* may encounter during infection.

Previous studies showed that *X. oryzae* pv. *oryzae*, *X. campestris* pv. *campestris*, and *Burkholderia* sp. produce multiple DSF family signals (Deng et al. 2010; He et al. 2010; Zhou et al. 2015). The relative proportions of these signals are influenced by culture medium composition. DSF is the dominant signal produced by *X. campestris* pv. *campestris* in nutrient-rich yeast extract broth medium, whereas BDSF is the dominant one in nutrient-poor XOLN medium (Zhou et al. 2015). The plant environment is poor in nutrients and the xylem fluid mainly contains carbohydrates, inorganic ions, and organic substances such as amino acids (Satoh 2006). The present study further confirmed that several plant-derived compounds promote BDSF biosynthesis. Thus, the predominance of BDSF over DSF in the infected tissues should be a consequence of nutrient availability. However, we could not rule out other in-plant factors that might affect DSF family molecule preference, since there is still a significant level of DSF (19.3 to about 26.7% of the total) present in the infected cabbage tissues (Fig. 7). This study, therefore, does not rule out the possibility that

DSF also plays a role in *X. campestris* pv. *campestris* pathogenesis in Chinese cabbage. Further insights into this point will be gained from the experimental analysis of the physiological concentration of both DSF and BDSF in the infected tissues and a more thorough analysis of the differential effect of BDSF and DSF on the expression of various QS-dependent genes in *X. campestris* pv. *campestris*.

DSF and BDSF at higher concentrations ($\geq 2 \mu\text{M}$) displayed similar effects on *engXCA* expression and protease activity in vitro; however, DSF is more active than BDSF at lower concentrations ($\leq 1 \mu\text{M}$) (Fig. 7). Since both signals induce *engXCA* expression and protease activity through the receptor RpfC (Fig. 8), DSF probably has higher affinity to RpfC than BDSF. This needs to be further investigated. To our knowledge, this study represents the first report directly showing the in-plant production of a pathogen QS signal. These findings are also consistent with several other reports showing that the major DSF family signals produced by *Burkholderia* species, *Xylella fastidiosa*, and *Pseudomonas aeruginosa* are the straight-chained fatty acids, *cis*-2-dodecenoic acid, tetradecenoic acid, or *cis*-2-decenoic acid, respectively (Beaulieu et al. 2013; Davies and Marques 2009; Deng et al. 2010). The identification of BDSF as the dominant in-plant QS signal provides not only a better understanding of the molecular interactions between *Xanthomonas* spp. and crucifer host plants but also suggests BDSF as the logical target for the design of strategies to interrupt *X. campestris* pv. *campestris* signaling during infection.

In this study, the strains $\Delta\text{rpfC}\Delta\text{rpfB}$ and ΔrpfB , respectively, were used to monitor the DSF family signals produced in culture and in the infected cabbage tissue. However, actual situations with the wild-type strain in planta may be different. First, since RpfB has been shown earlier to have high homology to fatty acid metabolism enzyme FadD (Zhou et al. 2015), it could also alter the balance of free fatty acid present in the strain. These mutants themselves may have an effect on growth, virulence, and even signal production. Second, previous studies in *Xanthomonas* spp. and other closely related bacteria have shown that the secreted signals can be modified (Zhou et al. 2017). It is also possible that the DSF signals produced in planta by these mutant strains are modified by the plant enzymes or microbial enzymes that are associated with endophytes. Collectively, in the actual situation during wild-type colonization, predominant DSF signal production and response might be different from what we deduced in this study. Further studies are needed to get an exact idea about the DSF production dynamics inside the plant.

MATERIALS AND METHODS

Bacterial strains and growth conditions.

X. campestris pv. *campestris* wild-type strain *XC1* and their derivatives were described previously (He et al. 2006b). Medium and bacterial strains used in the present study are listed in Supplementary Table S1. *X. campestris* pv. *campestris* strains were grown at 30°C in media with 25 μg of rifampicin per milliliter. For media preparation, tryptone, peptone, yeast extract, sucrose, amino acids, α -keto acids, carboxylic acids, phenolic acids, organic acids, and plant hormones were purchased from Sangon Biotech (Shanghai, China). Bacterial growth was determined by measuring OD₆₀₀.

Extraction, purification, and quantitative analysis of DSF family signals

in *X. campestris* pv. *campestris* culture supernatant.

Extraction and purification of DSF family signals in *X. campestris* pv. *campestris* culture supernatants were

performed as previously described (He et al. 2010). Briefly, *X. campestris* pv. *campestris* strains were grown for 24 h and, then, 20 ml of supernatant was collected by centrifugation at 3,800 rpm for 30 min (Heraeus Multifuge x1R, Thermo Scientific). The pH of the supernatants was adjusted to 4.0. An equal volume of ethyl acetate was then added to the supernatant for extraction for 30 mins. Ethyl acetate fractions were collected, and the solvent was removed by rotary evaporation. The residues were dissolved in 1 ml of methanol. The crude extract was then subjected to a 0.45 m Minisart filter unit and the collected filtrate was condensed to 0.5 ml. Extract (3 μ l) was then subjected to high-performance liquid chromatography with a C18 reverse-phase column (4.6150 mm; Agilent) and was eluted with water in methanol (23:77, vol/vol, 0.1% formic acid) at a flow rate of 1 ml/min in an Agilent Technologies 1260 Infinity system with DAD G1315D VL detector.

Protein extraction and Western blotting analysis of RpfF expression level.

X. campestris pv. *campestris* cultures were collected by centrifugation at 12,000 rpm for 10 min at 4°C. The pellets were resuspended in 200 μ l of bacterial protein extraction mixture composed of 1 ml of EasyLys bacterial protein extraction solution (Thermo Scientific), 2 μ l of lysozyme, and 2 μ l of DNase I. The protein extract was kept at room temperature for 10 min and was then centrifuged at 12,000 rpm for 10 min at 4°C. Supernatant (100 μ l) was transferred into a clean Eppendorf tube. Protein concentration was determined by spectrophotometer at OD₅₉₅. Western blotting analysis was performed as described by Green and Sambrook (2012). The blocked membrane was incubated with primary polyclonal anti-RpfF serum (Zhou et al. 2015) for 4 h at room temperature. After washing with a 0.05% Tween-20-containing PBS buffer, the blots were incubated with the secondary goat anti-rabbit immunoglobulin G (H+L)–horseradish peroxidase conjugate (Bio-Rad). The hybridization signal was detected using the Super Signal West Pico chemiluminescent substrate (Pierce).

Preparation of Chinese cabbage hydrolysate samples.

Hydrolyzed Chinese cabbage samples were prepared as previously described with modifications (Xu et al. 2010). Chinese cabbage leaves (300 g) were chopped and blended with 300 ml of water for 15 min. The sample was pretreated with NaOH (1% wt/vol) at a ratio 1:10 for 15 min in an autoclave. The solids were removed using a filtration cloth. The filtrate was then centrifuged at 16,000 $\times g$ for 10 min, to remove any insoluble particles, and was adjusted to 7.0. The extract was condensed to 100 ml on a rotary evaporator (Eyela, Tokyo Rikakikai Co.) at 40°C.

Infection of Chinese cabbage by the *X. campestris* pv. *campestris* rpfB mutant.

X. campestris pv. *campestris* rpfB mutant strain was grown, overnight, in YYS medium. The bacterial cells were then collected by centrifugation and were resuspended into YYS medium to an OD₆₀₀ of 0.1. Fully matured Chinese cabbage (Wongbok) leaves were used for infection. The bottom part (about 1 cm) of each leaf was cut off, and the resulting wound was immediately immersed in the above-mentioned bacterial suspension in a beaker. The cabbage and beaker were then transferred to a glass desiccator (Isolab) that was connected to a nitrogen cylinder. The leaves were subjected to a 5-atmospheric pressure (5 $\times 10^5$ Pa) for 10 min to promote *X. campestris* pv. *campestris* to get entry into the vascular system of the cabbage leaf. The treated leaves were then transferred into a tray with wet filter papers. The tray was wrapped with plastic film and kept in an incubator at 25°C with 80% humidity for a week. The bottom part (about 3 cm) of

each leaf was removed for DSF extraction and colony counting. For each treatment, 10 pieces of leaves were used and two independent experiments were performed.

Extraction and ultra-performance liquid chromatographic-time of flight-mass spectrometry (UPLC-TOF-MS) analysis of DSF family signals in the infected cabbage.

To extract DSF family signals produced by *X. campestris* pv. *campestris* inside infected Chinese cabbage, 500 g of infected leaves were collected, were cut into small pieces, and were then blended with 200 ml of sterile water in an electric juicer (HR2168/40, Philips) for 10 min. The mixture was adjusted to a pH of 4.0, using hydrochloric acid (5 N). The mixture was then transferred to a 5-liter flask with 500 ml of ethyl acetate and was extracted for 30 min by shaking at 200 rpm at room temperature. The ethyl acetate fractions were collected by centrifugation at 10,000 rpm for 10 min, and the solvent was removed by rotary evaporation to dryness. The resulting residue was dissolved in 2 ml of methanol and was subjected to flash column chromatography, using a silica gel column (12 $\times$ 150 mm, Biotage Flash 12M cartridge), and finally, was eluted with ethyl acetate-hexane (25:75, vol/vol, 0.05% acetic acid). The collected active component was condensed and applied to UPLC-TOF-MS (Agilent) for further analysis.

Detection and quantitative analysis of DSF and BDSF from the extract were conducted following the recently published protocol (Zhou et al. 2018). Briefly, 5 μ l of the collected samples was applied to an UPLC system (Agilent 1290 Infinity, Agilent Technologies) on a Zorbax XDB C18 reverse phase column (4.6 $\times$ 150 mm, 5 μ m, Agilent Technologies) and was eluted with methanol-water (80:20, vol/vol) at a flow rate of 0.4 ml/min. LC elute was coupled to an accurate mass TOF MS (Agilent TOF MS 6230, Agilent Technologies) equipped with an Agilent Jet Stream electrospray ionization source. The mass spectrometer was operated in the negative ion mode. Nitrogen was used for drying (8 liters per minute, 325°C), sheath (11 liters per minute, 350°C), and nebulizer gas (35 psi). To quantify the BDSF and DSF concentration in the sample, a standard curve for DSF or BDSF by plotting the peak area vs. the known concentrations (1, 5, 10, and 50 μ M). The concentration of DSF and BDSF in the extract is calculated as described by Zhou et al. (2018).

Quantitative determination of protease activity and quantitative real-time polymerase chain reaction (PCR) analysis of the *engXCA* mRNA level in *X. campestris* pv. *campestris* culture.

The extracellular protease activity in culture supernatants was quantitatively analyzed according to the method previously described by Swift et al. (1999). For quantitative real-time PCR analysis, total RNAs were isolated using the RNeasy mini-prep kit (Qiagen). The PrimeScript RT reagent kit was used for quantitative reverse transcription PCR analysis. The primer set designed from a *engXCA* full DNA sequence were as follows: Eng-FOR, TTCAACGACAGCAACTTCCC; Eng-REV, GACC AGTAGAAGCCCCTGGTT. Quantification of gene expression and melting curve analysis were performed using Mastercycler ep Realplex 4S (Eppendorf) and SYBR Premix Ex Taq (Takara). The 16S rRNA gene was used as a reference to standardize all the samples and replicates. For protease activity and *engXCA* level analyses, the strain was grown in YYS medium at 28°C with shaking at 200 rpm for 24 h.

Statistical analysis.

Analysis of variance for experimental datasets was performed using GraphPad prism software version 5.0 (GraphPad

software Inc.). Significant effects of different treatments were determined by the *F* value (*P* = 0.05). Each experiment was repeated twice, and the data was shown as mean with one standard deviation.

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