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ORIGINAL ARTICLE

SstF, a novel sulforaphane-sensing transcription factor of *Xanthomonas campestris*, is required for sulforaphane tolerance and virulence

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Abstract

Avoiding the host defence system is necessary for the survival of pathogens. However, the mechanisms by which pathogenic bacteria sense and resist host defence signals are still unknown. Sulforaphane (SFN) is a secondary metabolite of crucifers. It not only plays an important role in maintaining the local defence response but also directly inhibits the growth of some pathogens. In this study, we identified a key SFN tolerance-related gene, *saxF*, in *Xanthomonas campestris* pv. *campestris* (Xcc), the causal agent of black rot in crucifers. More interestingly, we found that the transcription of *saxF* was regulated by the novel transcription factor SFN-sensing transcription factor (SstF). As a LysR family transcription factor, SstF can sense SFN and regulate the expression of *saxF* cluster genes to increase SFN resistance by directly binding to the promoter of *saxF*. In addition, we found that SstF and *saxF* also play an important role in positively regulating the virulence of Xcc. Collectively, our results illustrate a previously unknown mechanism by which Xcc senses the host defence signal SFN and activates the expression of SFN tolerance-related genes to increase virulence. Therefore, this study provides a remarkable result; that is, during pathogen–plant co-evolution, new functions of existing scaffolds are activated, thus improving the proficiency of the pathogenic mechanism.

KEYWORDS
SstF, sulforaphane, transcription factor, virulence, *Xanthomonas campestris*

1 | INTRODUCTION

Plants are sessile organisms that are under constant attack from microbes (Newman et al., 2013). To survive in complex environments, plants have evolved sophisticated defence mechanisms that allow the detection of environmental cues and translate them into physiological responses (Aznar et al., 2015). Decades of studies have revealed a large number of secondary metabolites with proven or putative functions in plant responses to pathogenic microorganisms (Piasecka et al., 2015). Some secondary metabolites can not only work as signals to activate the plant immune response but also play an antimicrobial role. These compounds are commonly considered

Bo Wang and Zhizhou Xu contributed equally to this work.

to function in plant defence as in planta antibiotics and are also called phytoalexins (Ahuja et al., 2012), such as sulforaphane (Qi et al., 2018; Schillheim et al., 2018). Interestingly, even though plants have evolved sophisticated immune systems, some pathogens can still infect host plants, which means that the co-evolution of plants and their pathogens has given rise to elaborate attack–counterattack strategies (Chen et al., 2022), but how pathogens overcome the adverse effects of phytoalexins to cause plant disease is not yet fully understood.

Sulforaphane (SFN), a secondary metabolite of crucifers (Piasecka et al., 2015), was identified as an important plant defence-priming compound that plays an important role in maintaining the local defence response of *Arabidopsis* (Andersson et al., 2015). SFN is released by *Arabidopsis* leaf tissue undergoing a hypersensitive response (HR), and this compound induces cell death and primes defence in naive tissue (Andersson et al., 2015). In addition, SFN can not only regulate resistance gene expression in *Arabidopsis* by inducing covalent modification (H3K4me3 and H3K9ac) of histone H3 in the promoter and promoter-proximal regions of the defence genes *WRKY6* and *PDF1.2* (Schillheim et al., 2018), but also directly inhibit the growth of *Hyaloperonospora arabidopsidis*, *Xanthomonas campestris* (Xcc), and other pathogens (Fan et al., 2011; Schillheim et al., 2018; Wang et al., 2022). A recent study showed that SFN can enter bacterial cells and directly bind with some important virulence-related transcription factors, such as OxyR and HrpS, to inhibit the pathogenicity of bacterial pathogens (Wang et al., 2020, 2022). Interestingly, Fan et al. found that during pathogen–plant co-evolution, pathogenic *Pseudomonas* strains, such as *Pseudomonas syringae* pv. *maculicola* ES4326 and *P. syringae* pv. *tomato* (Pst) DC3000, have developed survival in *Arabidopsis* extract (*sax*) genes that help host *Pseudomonas* bacteria and even nonhost *Pseudomonas* bacteria grow in SFN-containing environments (Fan et al., 2011). Our previous study also found conserved *sax* gene clusters in the Xcc genome (Wang et al., 2022). However, the transcription mechanism of *sax* genes is still unknown.

LysR-type transcriptional regulators (LTTRs) belong to the largest family of one-component DNA-binding transcription factors in bacteria (Sainsbury et al., 2009). They comprise an N-terminal DNA-binding domain containing a winged helix-turn-helix-motif joined by a long linker helix, which is involved in oligomerization, to a C-terminal regulatory domain (Zaim & Kierzek, 2003). The regulatory domains of LTTRs share a similar fold with periplasmic substrate-binding proteins and constitute the sensor part of the molecule (Tyrrell et al., 1997), binding effector molecules or responding directly to redox-active compounds through the thiol groups of cysteine residues (Jo et al., 2015). LTTRs are dual function regulators acting as both autorepressors and activators of target promoters, frequently of genes colocated with the LTTRs in the chromosome (Schell, 1993). LTTRs regulate the expression of a wide variety of genes, including operons involved in amino acid metabolism, oxidative stress, degradation of aromatic compounds, and bacterial virulence (Jo et al., 2015; Sainsbury et al., 2009). Transcriptional activation of the target gene is commonly dependent on the interaction of the LTTR with an effector (Schell, 1993). However, most of the effectors interacting with LTTRs are still unknown.

In this study, we found a key SFN tolerance-related gene, *saxF*, in Xcc. In addition, we found that the transcription of *saxF* was regulated by the novel LysR family transcription factor SstF. SstF also plays an important role in mediating SFN tolerance and virulence. More interestingly, SFN can work as an active effector to induce the transcriptional activation of *saxF* by binding with SstF. Overall, our study uncovered the mechanism by which bacterial pathogens sense SFN and activate the transcriptional activation of SFN resistance-related genes. In addition, this study provides a remarkable example of how pathogens increase their proficiency in pathogenesis during pathogen–plant co-evolution.

2 | RESULTS

2.1 | *saxF* plays a key role in mediating the tolerance of Xcc to SFN

To clarify the mechanism by which pathogens perceive SFN and activate the expression of tolerance-related genes, we used Xcc, a causal agent of black rot of crucifers (Pandey et al., 2016), as a working model. Our previous study showed that there are four conserved *sax* genes in the Xcc genome (Wang et al., 2022), and they are located in different gene clusters, but the main gene responsible for maintaining SFN tolerance in Xcc remains unknown. In this study, we knocked out the four *sax* genes (*saxB*, *saxC*, *saxG/D*, and *saxF*) individually (Figure S1) by allelic homologous recombination methods in Xcc (Wang et al., 2021). To identify the key genes that mainly confer SFN tolerance in Xcc, the growth rates of Xc1 (wild type), Δ*saxB*, Δ*saxC*, Δ*saxG/D*, and Δ*saxF* in 100 μM SFN-containing NYG medium were measured. As shown in Figure 1, compared with growth without SFN, the growth rate inhibition ratio of Δ*saxF* was more than 90% when cells were grown in 100 μM SFN-containing NYG medium. The inhibition rates of Xc1, Δ*saxB*, Δ*saxC*, and Δ*saxG/D* reached approximately 50% (Figure 1b). In summary, *saxF* plays a key role in the SFN tolerance of Xcc.

2.2 | *saxF* is located in a conserved cotranscriptional cluster

To understand how *saxF* expression is controlled in Xcc, we performed genome sequence analysis of Xcc. As shown in Figure 2, *saxF* was located in a five-gene-containing cluster, and the five genes had the same transcription direction. Interestingly, we found the *Xcc1437* gene, which is located upstream of the *saxF* cluster and has the reverse transcription direction. Its encoded protein was annotated as a transcription factor. Here, we named this gene *sstF*. BLASTN analysis revealed that a relatively conserved region from *sstF* to *Xcc1442* was present in five *Xanthomonas* spp. and *Stenotrophomonas rhizophila* QL-P4 (Figure 2). Apart from *sstF*,

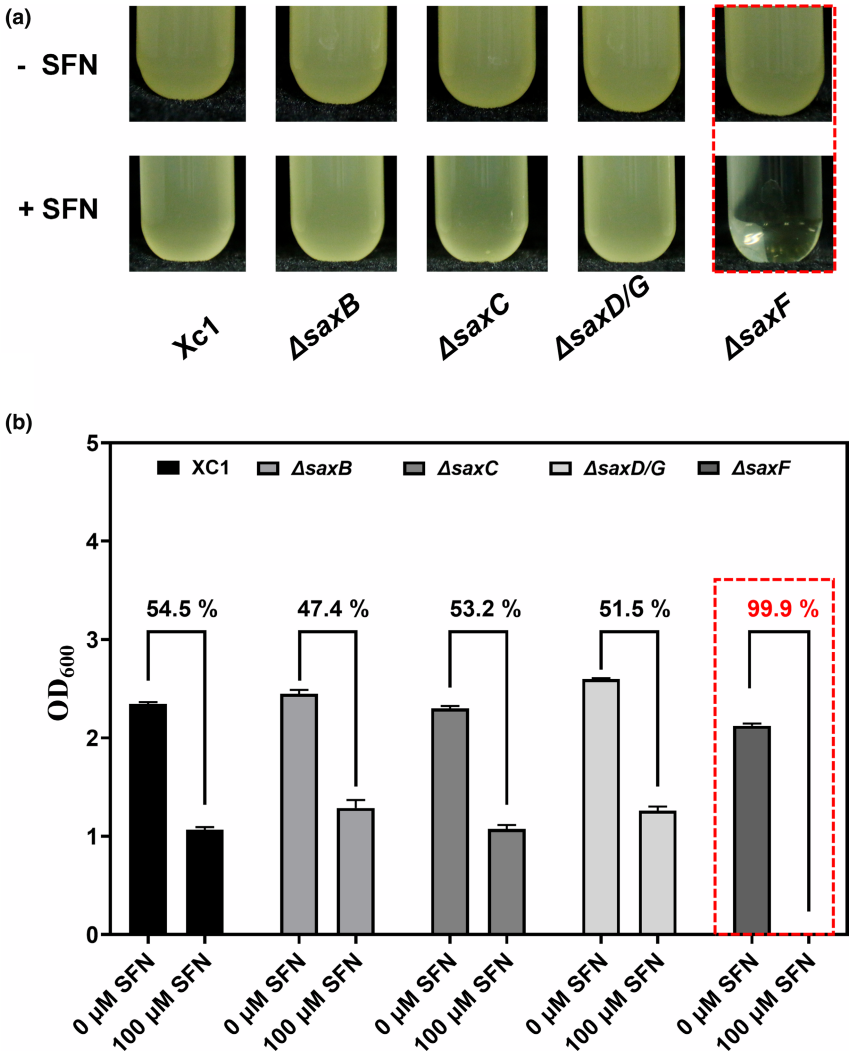


FIGURE 1 Identification of the key *sax* gene in *Xanthomonas campestris* pv. *campestris*. (a) The bacterial growth status of Xc1, Δ*saxB*, Δ*saxC*, Δ*saxD/G*, and Δ*saxF* in NYG medium with or without sulforaphane (SFN) at 28°C after 20 h. (b) The OD_{600} and growth rate inhibition ratio of Xc1, Δ*saxB*, Δ*saxC*, Δ*saxD/G*, and Δ*saxF* when grown in NYG medium with 100 μM SFN at 28°C for 20 h. The growth rate inhibition ratio was calculated by dividing the OD_{600} of bacteria grown in NYG medium with 100 μM SFN by that of bacteria grown in NYG medium without SFN. The bars are the mean ± standard deviation ($n = 9$). The data were obtained from triplicate experiments.

all the genes in this region shared the same transcriptional orientation. Reverse transcription (RT)-PCR analysis confirmed that *Xcc1438*, *Xcc1439*, and *saxF* belonged to the same operon in Xcc (Figure S2). Taken together, these results indicated that *saxF* was localized in a conserved cotranscription cluster, and the transcription of the genes in this cluster may share the same promoter and be regulated by a common transcription factor. More interestingly, a gene whose encoded protein was annotated as a transcription factor (*sstF*) was located in the conserved cluster in five *Xanthomonas* spp. and *S. rhizophila* QL-P4.

2.3 | SstF is required for full tolerance to SFN in Xcc

To determine whether SstF can act as a transcriptional regulator of *saxF* cluster genes, the *sstF* gene was successfully deleted using a homologous double-crossover protocol (Zhao et al., 2012). By testing the growth rate inhibition ratio as described in the Experimental Procedures section, we found that Δ*sstF*, Δ*sstF*(pBBR), and Δ*saxF* had the same weakened SFN tolerance (growth inhibition ratio of approximately 90%) (Figure 3). In addition, the complemented strain of Δ*sstF*(pBBR-*sstF*) showed complete recovery of SFN tolerance, which was similar to that of Xc1 (wild type). Overall, mutation of *sstF* could also decrease SFN tolerance, similar to mutation of *saxF*.

2.4 | In the presence of SFN, deletion of *sstF* weakens the transcription of *saxF* cluster genes

To further confirm whether SstF can regulate the expression of the *saxF* cluster, we determined the mRNA levels of *Xcc1438*, *Xcc1439*, *saxF*, and *sstF* in Xc1 and Δ*sstF* in the presence or absence of SFN. As shown in Figure 4, we found that the expression of *Xcc1438* (Figure 4a), *Xcc1439* (Figure 4b), and *saxF* (Figure 4c) was obviously induced by SFN in Xc1. In the Δ*sstF* strain, addition of SFN did not impact the expression of *saxF* cluster genes. Interestingly, the expression of *sstF* was not induced by SFN (Figure 4d). The results showed that SFN could induce the expression of all *saxF* cluster genes in the wild type, but SFN lost its inducer function in Δ*sstF*, indicating that SstF plays an important role in regulating the expression of *saxF* cluster genes.

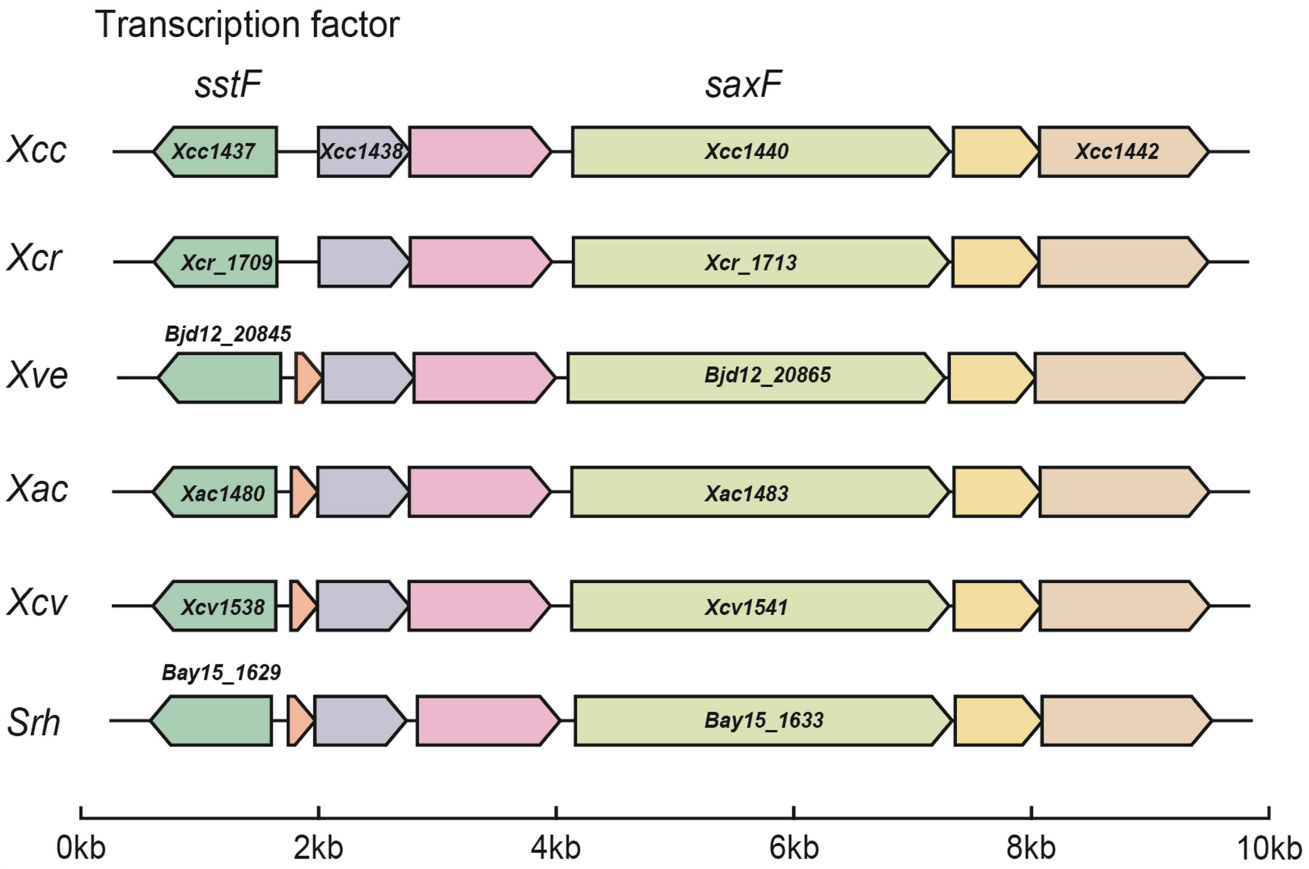


FIGURE 2 Genetic architecture of the *saxF*-containing cluster. Genetic architecture of the cluster containing six conserved genes in five *Xanthomonas* spp. and *Stenotrophomonas rhizophila* QL-P4. Xcc, *Xanthomonas campestris* pv. *campestris* ATCC33913; Xcr, *X. campestris* pv. *raphani* 756C; Xve, *Xanthomonas vesicatoria* ATCC35937 LMG911; Xac, *Xanthomonas citri* pv. *citri* 306; Xcv, *X. campestris* pv. *vesicatoria* 85-10; Srh, *Stenotrophomonas rhizophila* QL-P4.

2.5 | SstF, containing a DNA-binding domain and a putative effector-binding pocket, is a LysR family transcription factor

To determine the mechanism by which SstF regulates the expression of *saxF* cluster genes, we further performed detailed bioinformatics analysis of SstF. SstF is 344 amino acids in size, has a molecular weight of 37.98 kDa, and is a putative LysR family transcriptional regulator (https://www.uniprot.org/uniprotkb/Q8PAP2). We also analysed the three-dimensional structure of SstF using the Swiss-Model Repository program (http://swissmodel.expasy.org) and AlphaFold. When we searched for a template to build the three-dimensional model of SstF using the Swiss-Model Repository program, we found that the CrgA protein in *Neisseria meningitidis* has the highest identity (33.89%) with SstF, so we used the CrgA structure as a model (PDB: 3HHG, Figure 5b) (Sainsbury et al., 2009) and visualized it in PyMOL (Figure 5c). CrgA is a previously identified LTTR (Sainsbury et al., 2009). Sequence analysis showed that SstF and CrgA shared 33.89% identity, and the key arginine residue (R52) responsible for DNA binding in CrgA was also conserved in SstF (Figure 5a). Comparison of the protein structures of CrgA and SstF by TM-align (Zhang & Skolnick, 2005) showed that the length of the aligned residues was 207, the root mean square deviation between the aligned residues was 2.68, and the TM score normalized to the length of SstF was 0.547, indicating that the two proteins had approximately the same fold (Figure 5d). The above results indicate that the sequences and protein structures of CrgA and SstF are similar, and they both have a DNA-binding domain, a regulatory domain, and a putative effector-binding domain (Figure 5b–d), similar to other LysR family transcription factors (Schell, 1993; Tyrrell et al., 1997; Zaim & Kierzek, 2003). Therefore, SstF, similar to other homologous LysR family transcription factors, may bind to the promoters of target genes to regulate their expression when it senses a cofactor.

2.6 | SstF can directly bind to the promoter of the *saxF* cluster and SFN

To understand how *saxF* cluster gene expression is regulated in Xcc, we performed a detailed analysis of the common promoter of this cluster upstream of *Xcc1438* via the BPROM database (http://www.softberry.com/cgi-bin/programs/gfindb/bprom.pl) (Solovyev & Salamov, 2011). Through the BPROM database, we

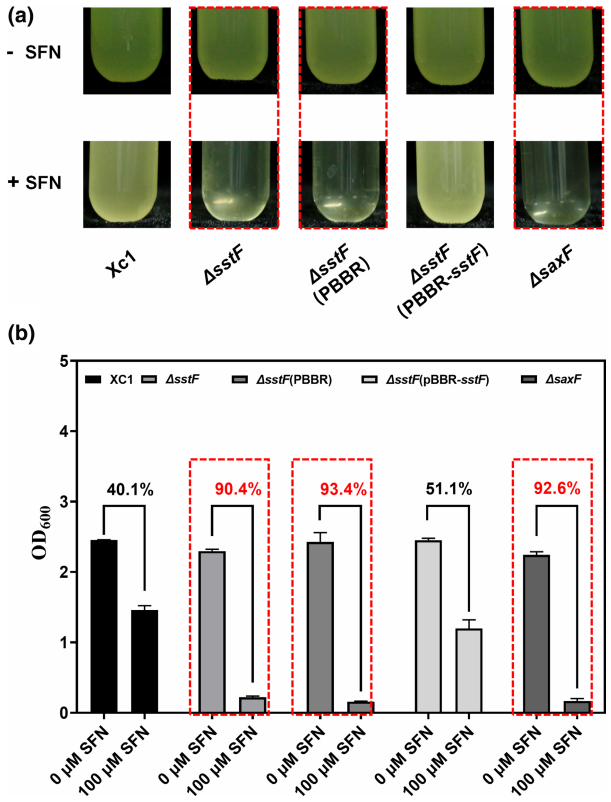


FIGURE 3 SstF is required for the sulforaphane (SFN) tolerance of *Xanthomonas campestris* pv. *campestris*. (a) The bacterial growth status of Xc1, Δ*sstF*, Δ*sstF*(pBBR), Δ*sstF*(pBBR-*sstF*), and Δ*saxF* in NYG medium with or without SFN at 28°C after 20 h. (b) The OD_{600} and growth rate inhibition ratio of Xc1, Δ*sstF*, Δ*sstF*(pBBR), Δ*sstF*(pBBR-*sstF*), and Δ*saxF* when grown in NYG medium with 100 μM SFN at 28°C for 20 h. The growth rate inhibition ratio was calculated by dividing the OD_{600} of bacteria grown in NYG medium with 100 μM SFN by that of bacteria grown in NYG medium without SFN at 28°C for 20 h. The bars are the mean ± standard deviation ($n = 9$). The data were obtained from triplicate experiments.

predicted the −10 and −35 sequences and located the transcription initiation site (TIS) of the *saxF* cluster (Figure S3). To test whether SstF controls *saxF* cluster transcription via direct binding to its promoter, an electrophoretic mobility shift assay (EMSA) was performed as described in the Experimental Procedures section. First, we cloned the putative promoter DNA fragments, covering approximately 250 bp upstream of the TIS of *Xcc1438*, named p-*saxF*. The subsequent addition of purified SstF-glutathione *S*-transferase (GST) protein (Figure 6a) at amounts ranging from 0 to 4 μg to the reaction mixtures (20 μl, 25°C, 10 min) containing 5 ng p-*saxF* caused a shift in the mobility of the p-*saxF* DNA fragments, suggesting that under these conditions, the SstF-GST protein was bound directly to p-*saxF* (Figure 6b). The physiological concentration of SFN in *Arabidopsis* apoplasts is approximately 15–40 μM (Wang et al., 2020), so we selected 20 μM SFN to mimic the physiological concentration in planta. When we added 20 μM SFN to the reaction mixtures, the combined shift caused by the binding between SstF-GST and p-*saxF* was the same as that in the absence of SFN (Figure 6b).

To verify the above results, we used an additional bacterial one-hybrid system to test the potential direct interaction between SstF and the *saxF* cluster promoter (p-*saxF*), as described in our previous reports (Xu et al., 2016). As shown in Figure 6c, on selective medium, we clearly observed that the test *Escherichia coli* strain containing both SstF and p-*saxF* grew well, similar to the positive control, whereas the negative control failed to grow. This result indicates that direct binding of SstF to p-*saxF* occurred under the test conditions. When we added 20 μM SFN to the selective medium, the tested *E. coli* strain containing both SstF and p-*saxF* still grew well (Figure 6c).

Through EMSA and a bacterial one-hybrid system, we showed that the SstF-GST protein could directly bind to p-*saxF* with or without SFN. As we mentioned above, SFN-induced gene expression depends on SstF. As a LysR family transcription factor, this protein contains a putative effector-binding pocket, leading us to hypothesize that SFN could directly bind to SstF. To verify this hypothesis, we conducted surface plasmon resonance (SPR) analysis to measure the possible binding between SFN and SstF. As shown in Figure 6d, SFN physically bound SstF-GST with a binding constant (K_d) of 1.73e−8 M, suggesting a strong binding force between SFN and SstF-GST. In the SPR assay, we also used the GST protein as a negative control and dimethyl sulphoxide (DMSO) as a blank control (Figure S4). We further predicted the key binding site of SFN and SstF. Based on computer-based molecular docking analysis using the multiple ligand simultaneous docking program, one SFN molecule probably binds to the phenylalanine (F) residue at position 299. When we mutated phenylalanine to alanine, $SstF_{F299A}$ could not strongly bind with SFN (K_d = 1.43e−4 M, Figure 7a). In addition, $SstF_{F299A}$ did not function as well as wild-type SstF in restoring the SFN tolerance of Δ*sstF* (Figure 7b). These data suggest that $SstF_{F299}$ is essential for binding to SFN and for SFN tolerance in Xcc.

In summary, these experiments proved that SstF regulates the expression of the *saxF* cluster by directly binding to the promoter in the presence of SFN. Interestingly, SFN could activate transcription through direct binding to SstF, but direct binding did not change the DNA-binding ability of SstF.

2.7 | SstF mediates the virulence of Xcc

As a bifunctional defence-priming compound, SFN can not only maintain the local defence response of plants (Schillheim et al., 2018) but also directly reduces the virulence and inhibits the growth of pathogens (Wang et al., 2022). When pathogens lose their SFN tolerance, their virulence towards the host may be weakened. To test this hypothesis, the virulence of Δ*sstF*, Δ*sstF*(pBBR), the complemented strain Δ*sstF*(pBBR-*sstF*), and Δ*saxF* was tested on a susceptible cabbage variety (*Brassica oleracea* 'Jingfeng No. 1') by the leaf-clipping method (Li et al., 2020), with the wild-type strain Xc1 serving as the control. Ten days after inoculation with a final concentration of OD_{600} = 1.0, lesions of comparable intensity appeared along the leaves

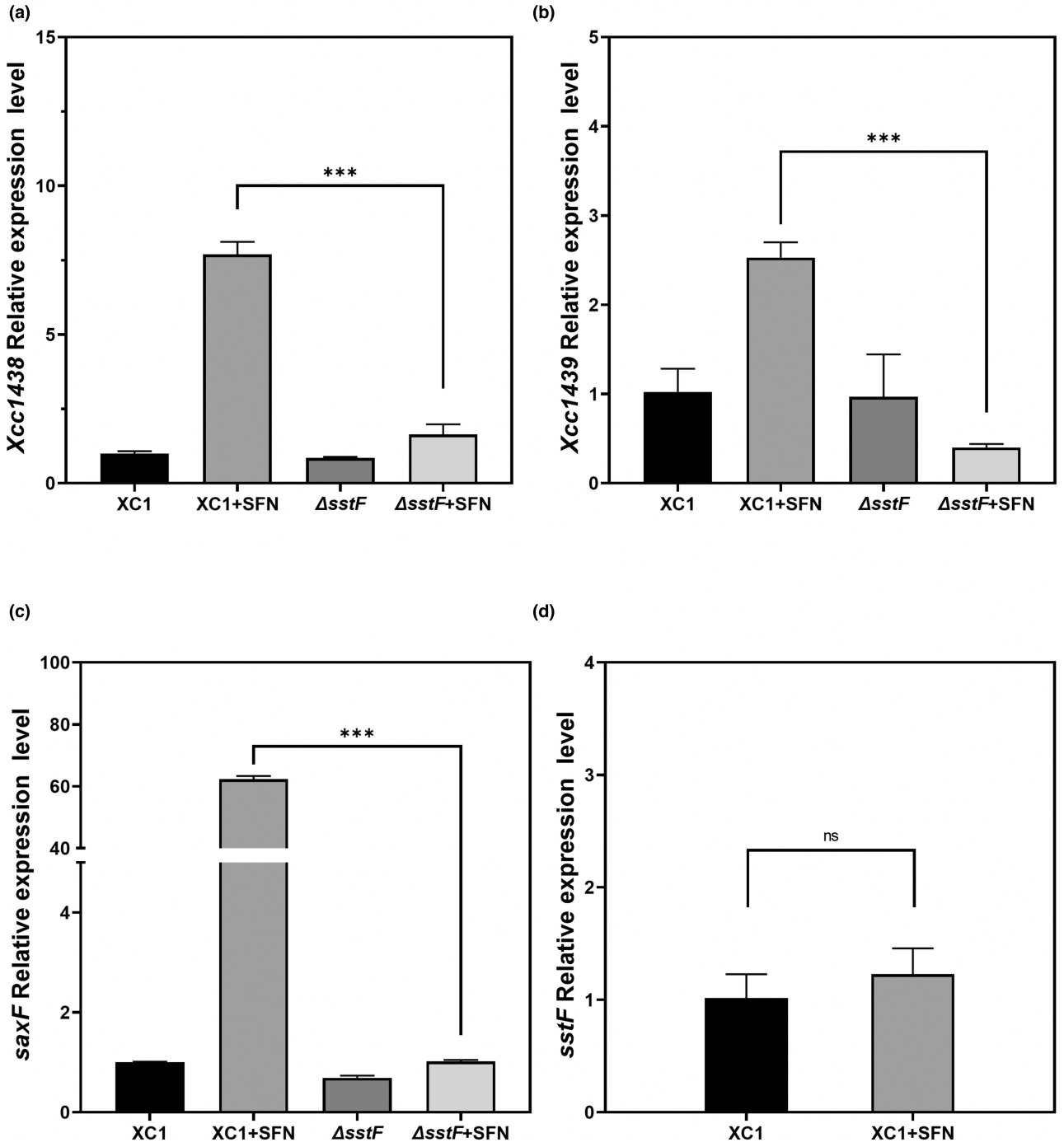
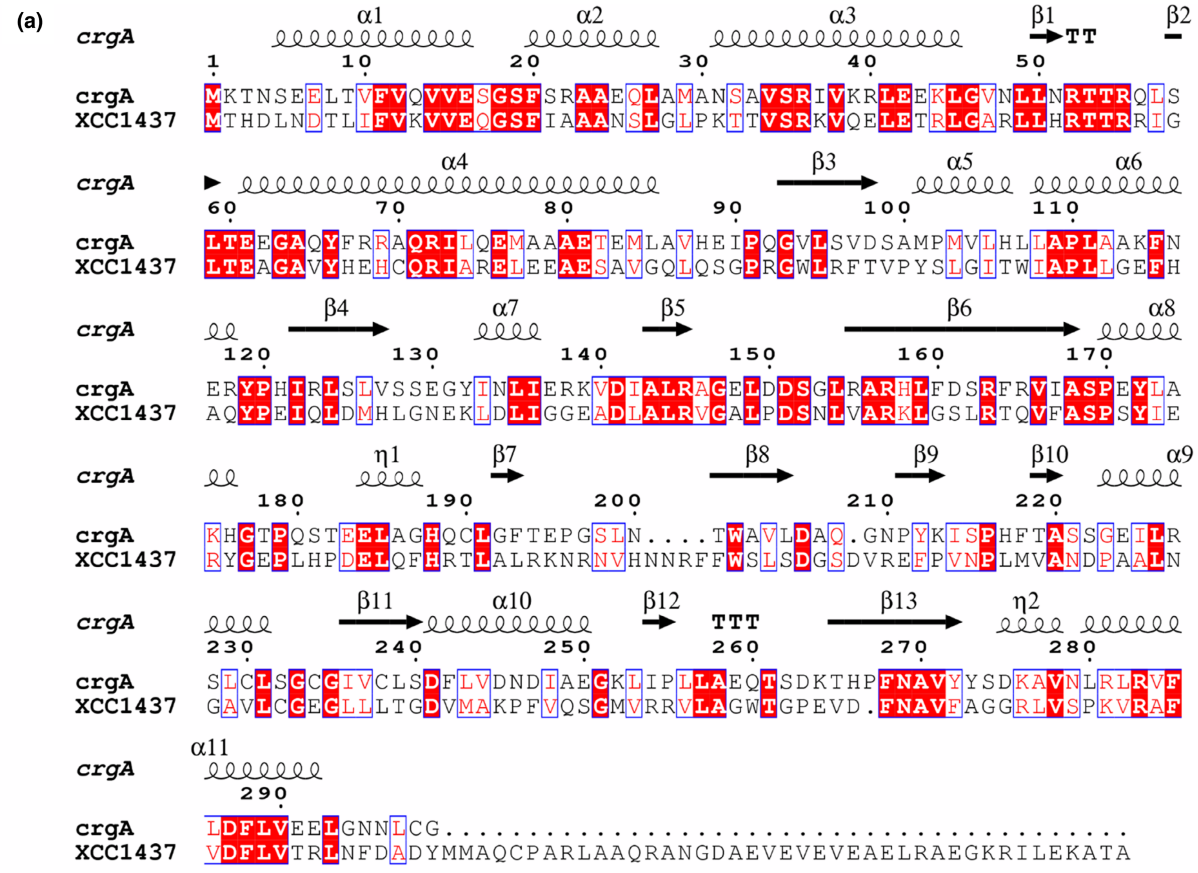


FIGURE 4 Analysis of *saxF* cluster genes and *sstF* expression in sulforaphane (SFN)-treated *Xanthomonas campestris* pv. *campestris* Xc1 or Δ*sstF* cells. (a–c) SFN led to a significant increase in the transcript levels of *Xcc1438* (a), *Xcc1439* (b), and *saxF* (c) in Xc1 but not in Δ*sstF*. (d) The expression of *sstF* could not be induced by SFN in Xc1. Xc1 or Δ*sstF* cells were incubated with 20 μM SFN for 6 h, and the transcript levels of *Xcc1438*, *Xcc1439*, *saxF*, and *sstF* were determined using reverse transcription-quantitative PCR. 16S rRNA was used as the endogenous control, and expression levels were normalized to the wild-type strain. Experiments were performed three times with similar results. Each column indicates the mean of three biologically independent experiments. Vertical bars represent standard errors. Statistical analysis was performed using two-way analysis of variance followed by Sidak's multiple comparison test. ***$p < 0.001$.

inoculated with either Xc1 or Δ*sstF*(pBBR-*sstF*), with no difference in lesion length ($p < 0.001$, *t* test). However, the lesions caused by Δ*sstF*, Δ*sstF*(pBBR), and Δ*saxF* were obviously shorter than those caused by Xc1 and Δ*sstF*(pBBR-*sstF*) (Figure 8a,b). The bacterial population in planta was also measured. After inoculation, the bacterial populations of Δ*sstF* and Δ*saxF* were markedly decreased in planta (Figure 8c). The complemented strain Δ*sstF*(pBBR-*sstF*) retained the wild-type colonization ability in planta (Figure 8c). This result indicated that *sstF* and *saxF* are required for the full virulence of Xcc.



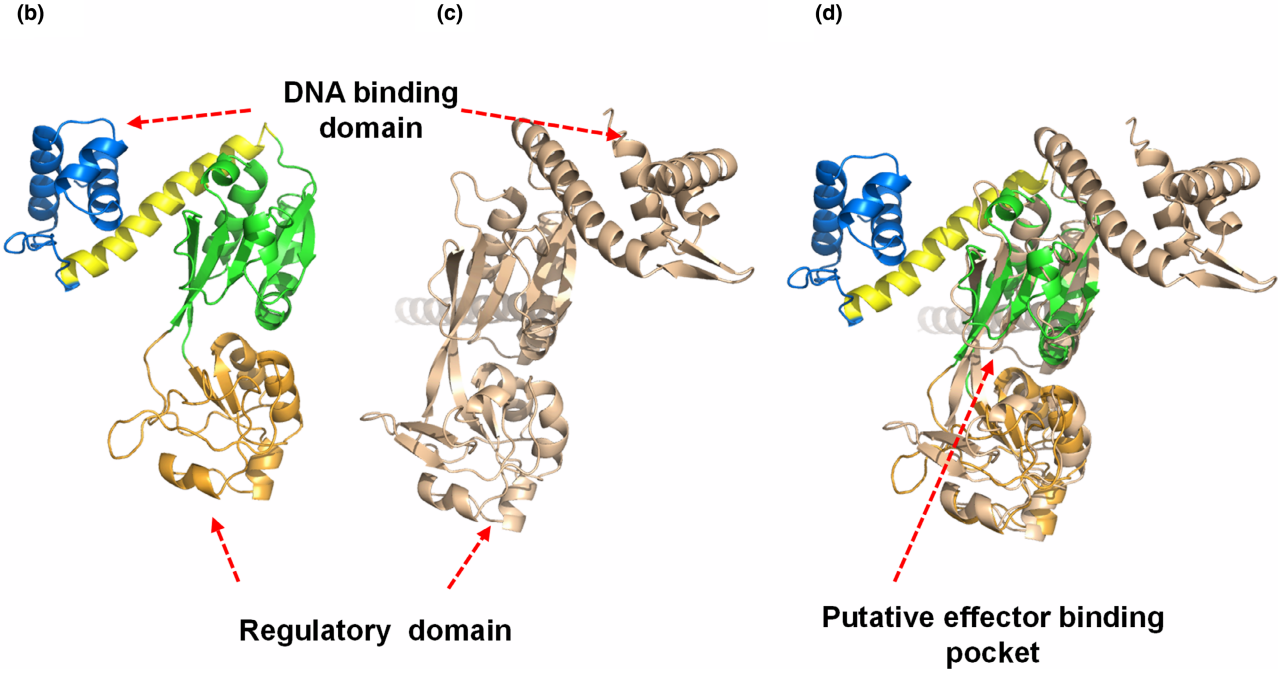


FIGURE 5 Protein sequence and structure alignment of SstF and CrgA. (a) Alignments were performed with ClustalW based on identical residues. The alignments were graphically rendered, and the structure of the *Neisseria meningitidis* CrgA structure as a reference (PDB ID: 3HHG) was superimposed using ESPript. Structural elements such as α-helices, turns (T), and β-strands (arrows) are indicated. Amino acid positions are indicated on top of the sequence. (b) CrgA fold and domain architecture were downloaded from PDB. (c) SstF fold and domain architecture, which were predicted by the Swiss-Model program and AlphaFold. (d) Structural alignment of SstF and CrgA by the TM-align database. The structure of SstF is similar to that of CrgA, with a Cα root mean square deviation value of 2.68.

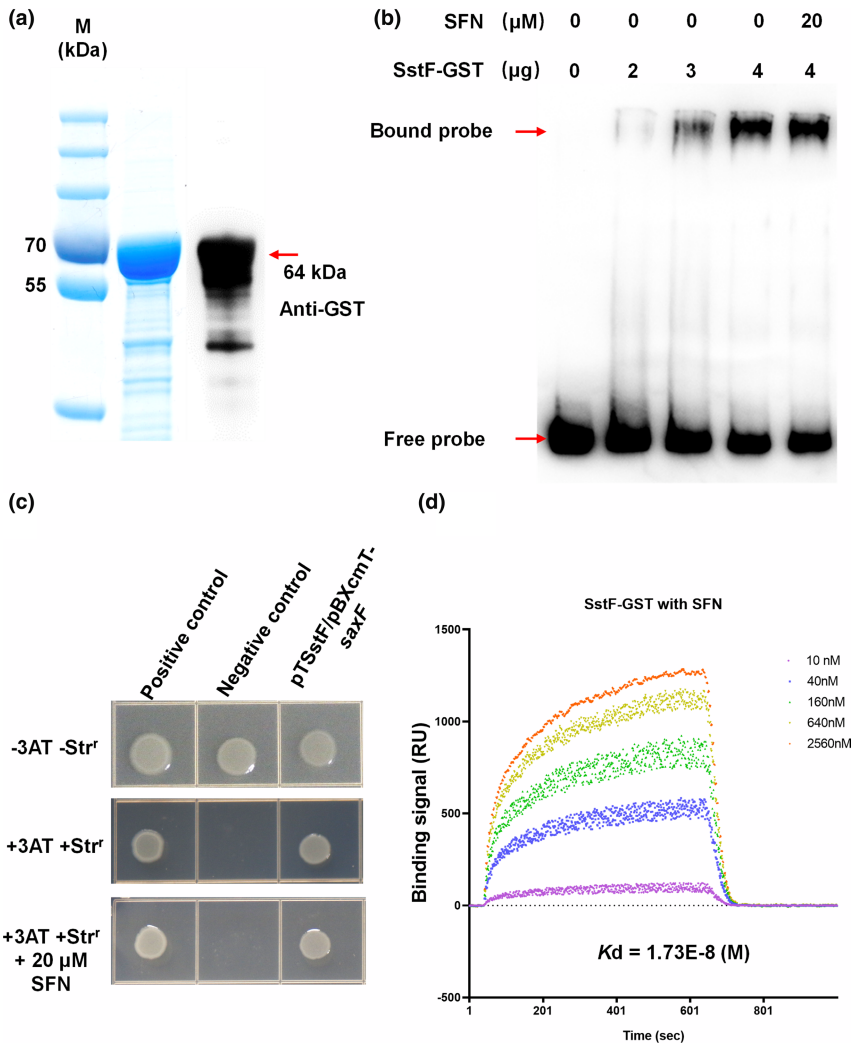


FIGURE 6 SstF can directly bind sulforaphane (SFN) and the promoter region of the *saxF*-containing cluster. (a) SDS-PAGE and western blot analysis of purified glutathione S-transferase (GST)-tagged SstF. (b) SstF binds to the promoter of *saxF* (p-*saxF*) in vitro. Electrophoretic mobility shift assay showing that SstF directly binds to p-*saxF*, and the formation of the complex could not be inhibited by adding 20 μM SFN. Different concentrations of SstF were added to reaction mixtures containing 20 ng of probe DNA, and the reaction mixtures were separated on polyacrylamide gels. (c) The direct physical interaction between SstF and the promoter region of *saxF* was detected in *Escherichia coli*. Positive control, co-transformant containing pBX-R2031 and pTRG-R3133; negative control, co-transformant containing pBXcmT-P*saxF* and empty pTRG; pTSstF/pBxcmT-*saxF*, co-transformant possessing both pTRG-SstF and pBXcmT-*saxF*; 3AT-Str^r (3AT, 3-amino-1,2,4-triazole; Str, streptomycin), nonselective Luria–Bertani (LB) medium plate; +3AT+Str^r, M9-based selective medium plate. (d) Surface plasmon resonance assay showing that SstF-GST forms a complex with SFN with a binding constant (K_d) of 1.73e−8 M.

3 | DISCUSSION

To avoid pathogen infection, plants have developed multiple defence strategies, and the use of secondary metabolites is an effective antipathogen strategy used by plants to fend off pathogen attack (Piasecka et al., 2015; Zhou & Zhang, 2020). To successfully infect hosts, pathogens have also evolved some strategies to overcome the defence mechanisms mediated by secondary metabolites (Chen et al., 2022; Qi et al., 2018). SFN is a secondary metabolite of crucifers, and it plays an important role in defence against pathogen attack. To survive in hosts that synthesize SFN, pathogens such as Pst DC3000 (Fan et al., 2011) and Xcc (Wang et al., 2022) have evolved an SFN resistance gene (*sax* gene). However, the mechanism by which bacterial pathogens sense SFN and regulate *sax* gene transcription is still unknown. In this study, we found that mainly *saxF* maintained SFN resistance, while *saxB*, *saxC*, and *saxD/G* did not (Figure 1). By genome sequence analysis, we found that *saxF* was located in a cotranscribed cluster, which was conserved in five *Xanthomonas* species and *S. rhizophila* QL-P4 (Figure 2). Interestingly, we found a new LysR family transcription factor, SstF, whose gene was located upstream of the *saxF* cluster. This transcription factor can sense SFN and regulate *saxF* cluster transcription by directly binding to the promoter (Figure 9). Our findings reveal the mechanism of bacterial pathogen perception and *sax* gene transcription regulation.

LTTRs comprise the largest family of prokaryotic transcription factors (Zaim & Kierzek, 2003). In response to different cofactors,

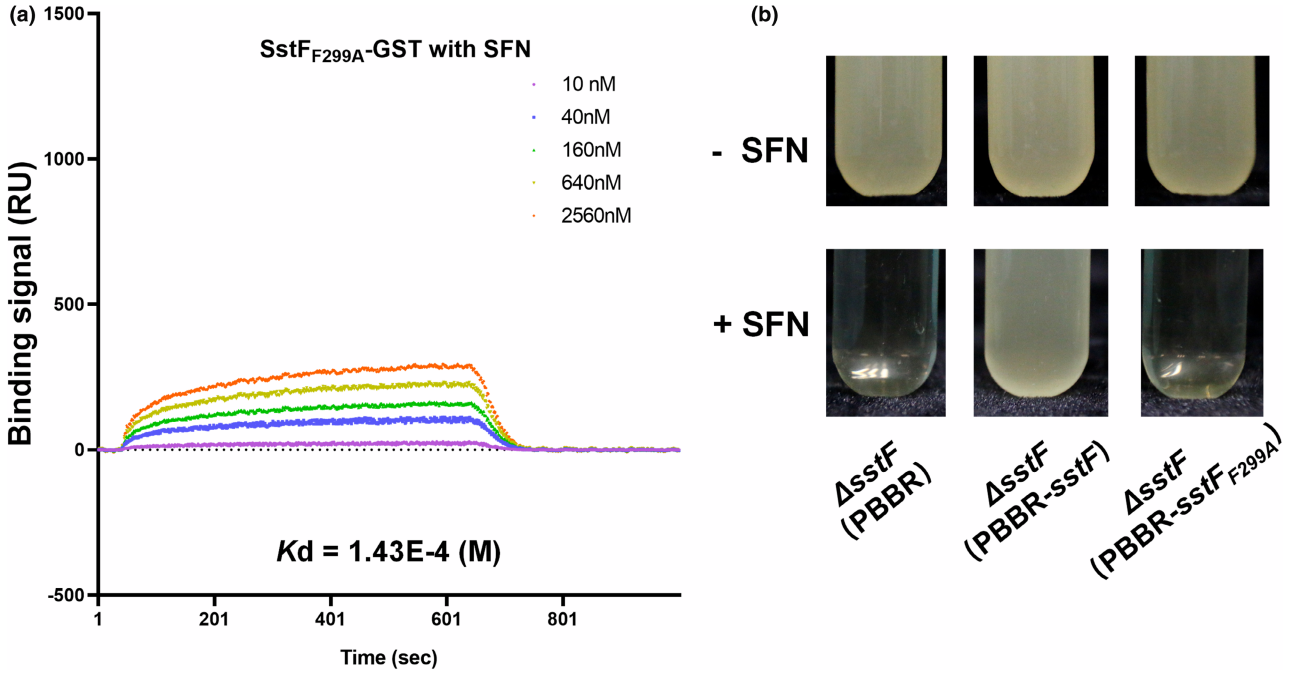


FIGURE 7 The binding site of sulforaphane (SFN) with SstF (phenylalanine at position 299) is essential for SFN tolerance of *Xanthomonas campestris* pv. *campestris*. (a) Surface plasmon resonance assay showing that the SstF$_{F299A}$-glutathione S-transferase (GST) fusion protein forms a complex with SFN with a very weak binding constant (K_d) of 1.43e−4 M. (b) The bacterial growth status of Δ*sstF*(pBBR), Δ*sstF*(pBBR-*sstF*), and Δ*sstF*(pBBR-*sstF*$_{F299A}$) in NYG medium with or without SFN at 28°C after 20 h.

these proteins activate divergent transcription of linked target genes or unlinked regulatory pathways with extremely diverse functions (Schell, 1993). To date, the function of some annotated LTTRs is still unknown, and most of the cofactors that induce transcriptional regulation have not been found. In this study, we identified a novel LTTR, SstF, that can sense SFN and regulate the transcription of *saxF* cluster genes. The sequence and structure of SstF share high identity with those of *N. meningitidis* CrgA (Sainsbury et al., 2009) (Figure 4). CrgA is an inducible LTTR of *N. meningitidis* that acts as both an autorepressor and an activator (Ieva et al., 2005). In this study, we found that SstF could directly bind to the promoter of the *saxF* cluster in the presence or absence of SFN, but only in the presence of SFN (Figure 6b,c) could SstF regulate the transcription of the *saxF* cluster (Figure 5). SPR analysis showed that SFN could directly bind to the SstF protein with a strong binding constant (K_d = 1.73e−8 M, Figure 6d), and SstF$_{F299}$ is essential for binding to SFN and SFN tolerance in Xcc (Figure 7a). These results suggest that SFN can function as a cofactor to induce the transcriptional regulation of SstF. Overall, our present study not only found a novel SFN resistance- and virulence-regulating LTTR, SstF, but also showed that SFN can function as a cofactor of SstF.

Most LTTRs, while activating the expression of target genes, repress their own expression, frequently by the use of divergent promoters (Zaim & Kierzek, 2003). In this study, we found that SFN could clearly induce the expression of *saxF* cluster genes but could not markedly change its own expression under the same conditions (Figure 5). Similar to other LTTRs, independent of the presence of the coinducer, SstF binds to the promoter region of the *saxF* cluster (Figure 6b,c) (Schell, 1993). Mutational studies have shown that coinducers can cause LTTR DNA-binding site changes that result in transcriptional activation (Schell, 1993). Some research has also indicated that coinducers can cause a conformational change in LTTRs (Sainsbury et al., 2009). In the present study, we did not determine how SFN activates transcriptional regulation. Whether SFN can change the DNA-binding site and the conformation of SstF will be examined in our future work by resolving the crystal structure of SstF and the SstF–DNA complex. In addition, transcription factors usually cooperate with a particular alternative sigma factor, so SFN may change the interaction between SstF and the specific sigma factor. LTTRs have been reported to regulate the expression of a wide variety of genes, including operons involved in amino acid metabolism, oxidative stress, degradation of aromatic compounds, and bacterial virulence (Sainsbury et al., 2009). In this study, we found that SstF, in addition to regulating SFN tolerance-related gene expression, can mediate virulence (Figure 8). However, whether the reduction in virulence of the *sstF* mutant was caused by weakened SFN tolerance or inhibition of the expression of other virulence-related genes is still unclear. In our future work, we will examine whether SstF can regulate the expression of other virulence-related genes by RNA sequencing and chromatin immunoprecipitation coupled with sequencing.

As described previously, Pst DC3000 (Fan et al., 2011) and Xcc (Wang et al., 2022) both harbour SFN resistance genes (*sax* genes). Pst DC3000 contains five SFN tolerance-related genes (*saxAB/F/D/G*), and deletion of each of these partially weakens SFN tolerance (Fan et al., 2011). Xcc contains all the homologous genes (Wang et al., 2022), and only deletion of *saxF* could change

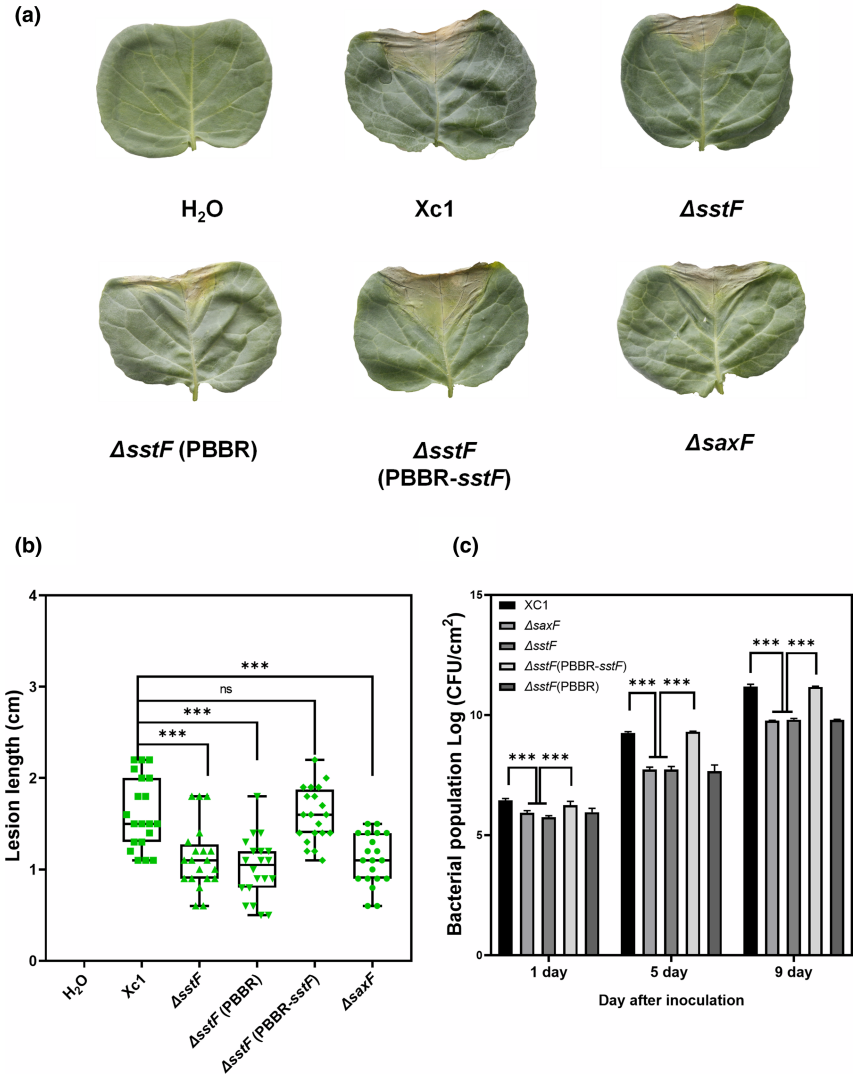


FIGURE 8 Deletion of *sstF* and *saxF* caused a deficiency in the virulence of *Xanthomonas campestris* pv. *campestris* (Xcc). (a) Bacterial strains were inoculated into leaves of the host plant *Brassica oleracea* 'Jingfeng No. 1'. Lesion length was estimated 10 days after inoculation. Sterile water was used as the negative control. (b) Lesion lengths of Xcc Xc1 and the derived strains on the plants shown in panel (a). The virulence of the Xcc strains was tested by measuring the lesion length after inoculating Jingfeng No. 1 with bacteria. The values are presented as the means and standard deviations of triplicate measurements, each for 20 leaves. Asterisks indicate significant differences relative to the Xcc Xc1 strain (unpaired *t* test; $^{***}p < 0.001$). (c) Bacterial counts in the top 2 cm^2 of each lesion-exhibiting leaf were scored. Error bar, mean ± standard deviation ($n = 3$). $^{***}p < 0.001$, assessed by one-way analysis of variance. All experiments were repeated three times with similar results.

SFN tolerance compared with that of the wild type (Figure 1). In our present work, we found that the transcription of *saxF* was regulated by SstF, but by BLAST analysis we did not find the SstF homologue protein in Pst DC3000. These results indicate that the SFN tolerance mechanisms of Pst DC3000 and Xcc may not be similar. During host–pathogen co-evolution, the directions for different pathogens may be different. In addition, as shown in Figure 5, the expression levels of cotranscribed genes in the *saxF* cluster were different, and *saxF* was expressed at higher levels with SFN. Some published papers have reported that some cotranscribed genes also have their own promoters (Slater et al., 2000; Wang et al., 2004). Here, we hypothesized that *saxF* may have other promoters and that its expression is controlled in more than one manner. These complex transcription mechanisms help the pathogen co-evolve with host plants.

In conclusion, in the present study, we not only identified a novel SFN tolerance mechanism and the virulence regulator SstF, but also dissected the mechanism by which Xcc senses the host defence signal SFN and activates the expression of virulence- and stress resistance-related genes. This study will assist future studies examining the co-evolution between hosts and pathogens.

4 | EXPERIMENTAL PROCEDURES

4.1 | Strains, plasmids, and growth conditions

The bacterial strains and plasmids used in this study are listed in Table S1. The *Xanthomonas* species were cultured in NYG medium (5 g/L tryptone, 3 g/L yeast extract, 20 g/L glycerol; pH 7.0) at 28°C. *E. coli* strains were grown in Luria–Bertani (LB) medium (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl; pH 7.0) at 37°C. When needed, kanamycin (Km, 50 μg/mL), ampicillin (Amp, 100 μg/mL), rifampicin (Rif, 50 μg/mL), and gentamycin (Gm, 25 μg/mL) were added to the growth medium for selection.

4.2 | Plant material and bacterial virulence assays

The plant material and bacterial virulence assays have been described previously (He et al., 2009; Li et al., 2020). Briefly, the susceptible cabbage cultivar *B. oleracea* 'Jingfeng No. 1' was grown in a growth chamber using a cycle consisting of 12 h of light at 25°C and 12 h of darkness at 23°C, with approximately 70% relative humidity. Plants

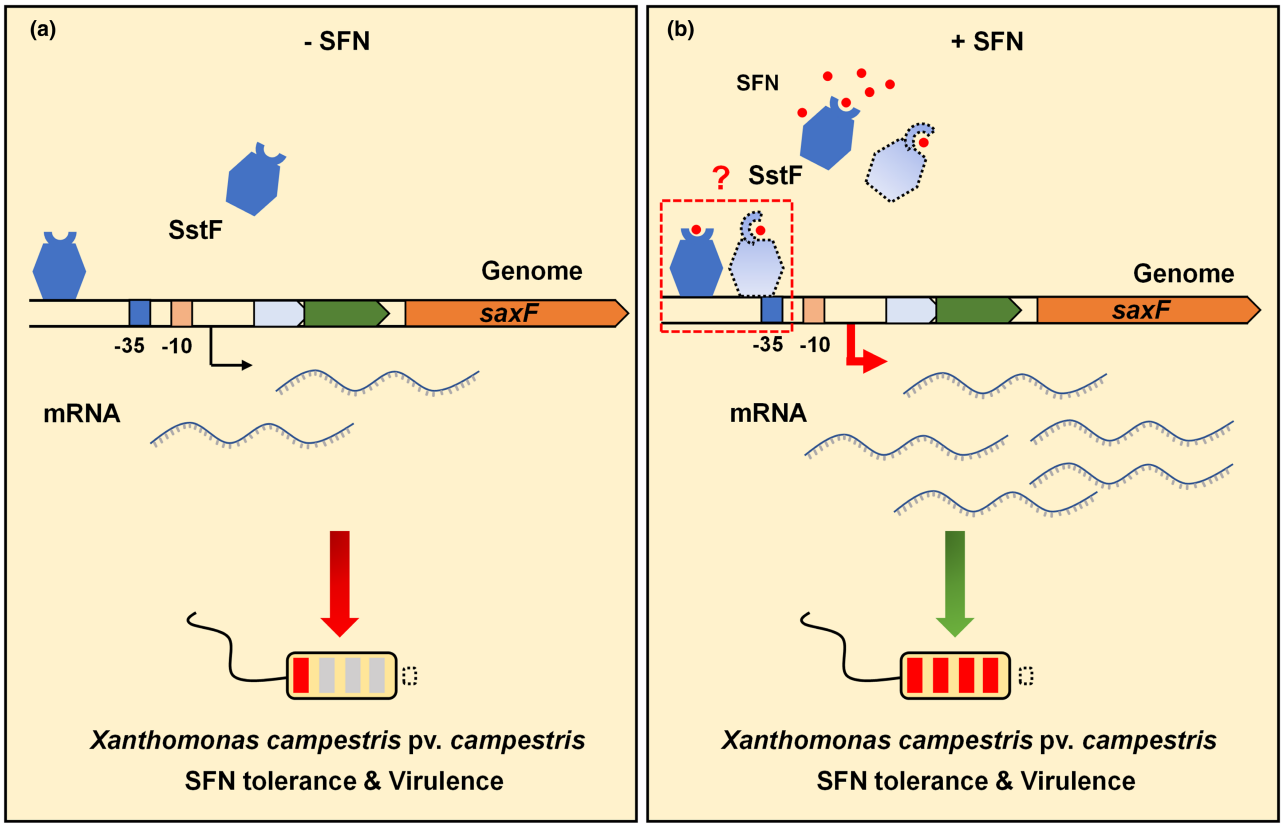


FIGURE 9 A schematic working model for SstF regulating the expression of *saxF* cluster genes. (a) SstF is a novel LysR family transcriptional regulation factor, and the transcriptional regulatory activity of SstF needs to be activated by sulforaphane (SFN). In the absence of SFN, the *saxF*-containing cluster is transcribed at a low level. SstF binds to the promoter of *saxF*. In this state, SstF is not activated, and bacteria lose their ability to adapt to SFN stress and decrease their virulence on host plants ("battery low"). (b) In the presence of SFN, SFN directly binds to OxyR, increasing the transcriptional regulatory activity of SstF, and the expression of the key SFN tolerance-related gene *saxF* is regulated by SstF, which directly binds to its promoter. In this state, bacteria are fully able to adapt to SFN stress and maintain full virulence on host plants ("battery fully charged"). However, how SFN activates SstF is still unknown. We have two hypotheses, which are shown in the red box. SFN may lead to a conformational change in SstF or change its DNA-binding site, thus activating SstF.

were inoculated at 6 weeks with bacterial suspensions at an approximate OD_{600} of 0.1 in sterile distilled water by immersing scissors in the freshly prepared bacterial suspensions and clipping approximately 0.5 cm from the tips of fully expanded leaves. Lesion lengths were measured 10 days after the inoculation of 20 leaves with each strain tested.

4.3 | Generation of mutants and complemented strains

Generation of the in-frame deletion mutant was conducted using wild-type Xc1 as the parental strain via allelic homologous recombination (Li et al., 2021; Wang et al., 2018; Wu et al., 2021). The 500-bp DNA fragments flanking the Xcc *sstF* and *sax* genes were amplified with *Pfu* DNA polymerase using Xc1 genomic DNA as a template and the corresponding primer pairs (Table S2). Fragments were purified and ligated into pK18mobsacB with the SE Seamless Cloning and Assembly Kit (ZOMANBIO) to obtain the plasmids pK18*mobsacB-saxB*, pK18*mobsacB-saxC*, pK18*mobsacB-saxD/G*, pK18*mobsacB-saxF*, and pK18*mobsacB-sstF*. The resulting constructs were transferred into Xc1 by electroporation, and Km was used to select for integration of the nonreplicating plasmid into the recipient chromosome. A single-crossover integrant colony was spread on NYG medium without Km at 28°C for 36 h, and after appropriate dilution, the culture was spread on NYG plates containing 10% sucrose. Colonies sensitive to Km were screened by PCR using the primers listed in Table S2, and the related deletion strains were obtained.

For complementation of the Δ*sstF* mutants, the coding regions of *sstF* were amplified by PCR and cloned into the versatile pBBR1MCS5 plasmid as described previously (Su et al., 2017; Yu et al., 2018). The resulting plasmid, pBBR-*sstF*, was transferred into the Xcc strain by electroporation. The Δ*sstF*(pBBR-*sstF*) strains were thus obtained.

4.4 | Protein expression and purification

Protein expression and purification were performed as described previously (Wang et al., 2021, 2022). Briefly, the coding regions of *sstF* (*Xcc1437*) were amplified by PCR using the corresponding

primer pairs (Table S2). The PCR product was purified, digested, and cloned into pGEX-6p-1, creating the final construct pGEX-6p-1-*sstF*. These vectors were transformed into *E. coli* BL21 (DE3) for protein expression. Briefly, the transformed strain was cultivated in LB medium containing 100 μg/mL Amp overnight at 37°C. Then, a 5-mL overnight culture was transferred into 500 mL of fresh LB medium containing 100 μg/mL Amp and grown at 37°C with shaking at 220 rpm until OD_{600} = 0.4. Subsequently, isopropyl β-D-1-thiogalactopyranoside was added to the culture at a final concentration of 0.2 mM, followed by further incubation at 28°C for 4 h. Then, the cells were collected by centrifugation (3381 × *g*) at 4°C and resuspended in 15 mL of phosphate-buffered saline (PBS) supplemented with phenylmethylsulfonyl fluoride at a final concentration of 1 mM for lysis. The cells were lysed by brief sonication, and the crude cell extracts were centrifuged at 7000 × *g* and 4°C. Soluble protein fractions were collected and mixed with glutathione Sepharose 4B (GE) for 2 h at 4°C before being placed into a column and extensively washed with PBS. The proteins were subsequently eluted using elution buffer containing reduced glutathione. Protein purity was assessed using SDS-PAGE, and the protein concentration was determined using a Bradford protein assay kit (Bio-Rad).

4.5 | RT-quantitative PCR assay

The mRNA levels of target genes were determined using RT-quantitative PCR (RT-qPCR) as described previously (Deng et al., 2018). Bacterial cells were grown in NYG medium until the OD_{600} reached 1.0, and then 0 μM or 20 μM SFN was added to the medium. After 6 h of culture, bacterial cells were collected. Total RNA was isolated by the E.Z.N.A. Bacterial RNA Kit (Omega Bio-Tek) according to the manufacturer's instructions. A NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific) was used to evaluate the RNA concentration and purity. The eluted RNA samples were treated with ribonuclease inhibitors and DNase I (Omega) to remove genomic DNA. RNA integrity was examined by electrophoresis on 1% agarose gels. A 2-μg aliquot of each RNA sample was used for complementary DNA synthesis with the PrimeScript RT Reagent Kit with Genomic DNA Eraser (TaKaRa). qPCR was performed with TransStart Top Green qPCR Super-Mix (TransGen Biotech) and a QuantStudio 6 Flex Real-Time PCR System (Applied Biosystems) with the following thermal cycling parameters: denaturation at 95°C for 30 s, followed by 40 cycles of 95°C for 5 s and 60°C for 34 s. Gene expression was analysed using the $2^{-\Delta\Delta Ct}$ method with 16S ribosomal RNA serving as the endogenous control and normalized to the expression level in the wild-type strain. The experiments were performed three times, each involving three replicates.

4.6 | SFN tolerance assay

The SFN tolerance of different Xcc strains was tested by turbidimetry (Shi et al., 2015). Bacterial cells were grown in NYG medium until the OD_{600} reached 1.0, and then approximately 250 μL of the NYG medium containing bacterial cells was transferred to a new bottle containing 25 mL NYG medium with 0 or 100 μM SFN. The inoculated test bottles were incubated at 28°C and continuously shaken at 180 rpm for 20 h until the bacteria reached the logarithmic growth phase. The growth of the cultures was monitored on a BioPhotometer plus (Bio-Rad) by measuring the OD_{600}. The growth inhibition rate *I* was calculated by the following formula:

$$I(\%) = (C - T)/C \times 100,$$

where *C* is the corrected OD_{600} of bacterial growth on untreated NYG (0 μM), *T* is the corrected turbidity value of bacterial growth on treated NYG (100 μM), and *I* is the growth inhibition rate.

4.7 | Bacterial one-hybrid assay

The bacterial one-hybrid reporter system was used to examine the potential interaction between SstF and the promoter of *saxF*. According to our previous work (Wang et al., 2018; Xu et al., 2016), the bacterial one-hybrid reporter system contained three components: the plasmids pBXcmT and pTRG, which were used for cloning the target DNA and expressing a target protein, respectively, and the *E. coli* XL1-Blue MRF′ *Kan* strain, which was the host strain for propagating the pBXcmT and pTRG recombinants. The *saxF* promoter region (250 bp) was cloned into pBXcmT, generating the recombinant vector pBXcmT-*saxF*. Similarly, the coding region of *SstF* (1,035 bp) was cloned into pTRG, creating the final construct pTRG-SstF. The two recombinant vectors were transformed into the XL1-Blue MRF′ *Kan* strain. If direct physical binding occurred between SstF and the *saxF* promoter, the positive transformant containing both pBXcmT-*saxF* and pTRG-SstF grew well on selective medium, which was minimal medium containing 5 mM 3-amino-1,2,4-triazole, 8 μg/mL streptomycin, 12.5 μg/mL tetracycline, 34 μg/mL chloramphenicol, and 30 μg/mL Km, as described previously. To test whether SFN affects the binding between SstF and the promoter of *saxF*, we also prepared a selective medium with 20 μM SFN. The co-transformant containing pBX-R2031/pTRG-R3133 served as a positive control, while the co-transformant containing the empty pTRG and pBXcmT-*saxF* was used as a negative control. All the co-transformants were spotted onto selective medium, grown at 28°C for 3–4 days, and then photographed.

4.8 | EMSA

EMSAs were performed as described previously (Wang et al., 2018). The *saxF* promoter region (250 bp) was amplified by PCR using the 5′-biotin-labelled primers p-*saxF*-F/R (Table S2). EMSA was carried out using the LightShift EMSA Optimization & Control Kit (Thermo) as recommended by the manufacturer with some modifications. In brief, 5 ng of the biotin-labelled probe and a series of SstF-GST

fusion protein concentrations (0 to 4 μg) were added to the reaction solution. SFN (20 μM) was included as appropriate in this assay. After incubation at 25°C for 10 min, the products were loaded onto a native 8% (wt/vol) polyacrylamide gel, electrophoresed in 0.5× TBE buffer for approximately 1.5 h at 100 V, and then transferred to a nylon membrane (Millipore). Protein–DNA complexes were visualized by VersaDoc (Bio-Rad).

4.9 | SPR assays

The binding kinetics of the SFN–SstF interaction were examined by the SPR assay, which was performed using the bScreen LB 991 Label-free Microarray System (BERTHOLD TECHNOLOGIES). SFN was fixed on the chip by photocrosslinking. Then, the SstF-GST fusion protein at different concentrations (10 nM, 40 nM, 160 nM, 640 nM, and 2.56 μM) was injected sequentially into the chamber in PBS with 0.1% Tween 20 (PBST, pH 7.4). The reaction temperature was controlled at 4°C, the binding time was 600 s, the disassociation time was 360 s, the flow rate was 0.5 mL/s, and the chip was regenerated with glycine hydrochloride (pH 2.0). GST and DMSO were used as negative and blank controls, respectively.

The raw sensorgrams and measurements of the binding process of ligands and proteins were recorded in real time. The response unit of surface resonance was compared to determine the different binding affinities between each sample dot. The response unit data collected on the SPR biosensor were further processed to eliminate any artefact, such as nonspecific binding and differences in buffer composition. Analysis of association and dissociation rate constants (k_a/k_{on} and k_d/k_{off}, respectively) and the equilibrium dissociation constant (K_D, k_d/k_a) was performed using the data analysis software of the bScreen LB 991 unlabelled microarray system according to a single-site binding model (1:1 Langmuir binding) with mass transfer limitations for binding kinetics determination.

4.10 | Bioinformatics analyses

The homologous cluster of *saxF* located in other bacteria was identified from the KEGG database (https://www.genome.jp/kegg/) by BLASTN. The gene clusters from different bacteria were compared by the ChiPlot web tool (https://www.chiplot.online/). The three-dimensional model of SstF was prepared using the Swiss-Model Repository program (http://swissmodel.expasy.org) (Waterhouse et al., 2018) and AlphaFold (Jumper et al., 2021) with the *N. meningitidis* CrgA structure as a reference (PDB: 3HHG). The CrgA structure was downloaded from the RCSB Protein Data Bank (PDB) (https://www.rcsb.org/). The protein sequences of SstF and CrgA were downloaded from UniProt (https://www.uniprot.org/). Protein sequence alignment between SstF and CrgA was performed by the Clustal Omega online service (https://www.ebi.ac.uk/Tools/msa/clustalo/) and ESPript 3.0 (https://espript.ibcp.fr/ESPript/cgi-bin/ESPript.cgi) (Robert & Gouet, 2014). Protein structural alignment between SstF and CrgA was performed by TM-align (https://zhanggroup.org/TM-align/) (Zhang & Skolnick, 2005). Prediction of the isoelectric point and the molecular weight of the selected proteins was performed using the ExPASy tool (https://www.expasy.org/). The promoter sequence and its characteristics were predicted by the BPROM database (http://www.softberry.com/berry.phtml?topic=bprom&group=programs&subgroup=gfindb) (http://www.softberry.com/cgi-bin/programs/gfindb/bprom.pl) (Solovyev & Salamov, 2011).

4.11 | Statistical analysis

The experimental datasets were subjected to analysis of variance (ANOVA) using GraphPad Prism v. 8.0 software. The statistical analyses and the exact values of *n* are described in detail in the figures and figure legends.

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CONFLICT OF INTEREST STATEMENT

None of the authors have financial or other potential conflicts of interest to declare.

DATA AVAILABILITY STATEMENT

The data that supports the findings of this study are available in the supplementary material of this article.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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