INTRODUCTION

In the natural environment, plants coexist in an intimate relationship with a variety of microbial pathogens, such as fungi, oomycetes, bacteria, and viruses. Plants must recognize the invaders and activate fast and effective defence mechanisms that arrest pathogen growth to ward off these pathogens. The failure of many potential parasites to colonize different plant species indicates the robustness of plant...
immune systems. To date, major advances in our understanding of plant innate immunity include the discovery of the mechanisms underlying pathogen recognition and the downstream signalling pathway and transcriptional activation of defence-related genes (Jones & Dangl, 2006; Li et al., 2016; Web et al., 2012). In contrast to these significant insights, relatively little is known about the processes that are directly responsible for the cessation of pathogen growth and virulence (Piasecka et al., 2015). Based on accumulating experimental evidence, plant secondary metabolites contribute to the inhibition of the infection process (Gleadow & Møller, 2014; Halkier & Gershenson, 2006; Mugford et al., 2013). Phytoalexins are inducible secondary metabolites with antimicrobial activity toward phytopathogens (Ahuja et al., 2012). However, the pathogen-inhibiting mechanism of some of the important phytoalexins is still unknown.

Sulforaphane (SFN), a secondary metabolite of crucifers, is an isothiocyanate derived from glucoraphanin (Piasecka et al., 2015). Recently, SFN was identified as a novel plant defence-priming compound that directly inhibits the growth of *Hyaloperonospora arabidopsidis* and other pathogens. Additionally, SFN also regulates resistance gene expression in *Arabidopsis* by inducing covalent modification (H3K4me3 and H3K9ac) of histone H3 in the promoter and promoter-proximal region of the defence genes *WRKY6* and *PDF1.2*, which primes *WRKY6* expression and up-primes *PDF1.2* activation to reduce susceptibility to *H. arabidopsidis* (Schillheim et al., 2018). In addition, SFN plays an important role in maintaining the local defence response of *Arabidopsis*: SFN is released by *Arabidopsis* leaf tissue undergoing a hypersensitive response (HR), and induces cell death and primes defence in naive tissue (Andersson et al., 2015). In addition to regulating *Arabidopsis* defence against host pathogen growth, it inhibits the growth of nonhost *Pseudomonas* bacteria in *Arabidopsis* plants (Fan et al., 2011). Pathogenic *Pseudomonas* strains, such as *Pseudomonas syringae* pv. *maculicola* (Psm) ES4326 and *P. syringae* pv. *tomato* (Pst) DC3000, possess survival in *Arabidopsis* extract (sax) genes that help host *Pseudomonas* bacteria and even non-host *Pseudomonas* bacteria grow in SFN-containing environments (Fan et al., 2011). Recently, SFN was found to directly target the key transcription-controlling factor HrpS to inhibit type III secretory system gene expression and decrease the virulence of Pst DC3000 (Wang et al., 2020). However, researchers have not yet determined whether and how SFN regulates the growth or virulence of other bacterial pathogens.

Harnessing the toxic properties of reactive oxygen species (ROS) to combat invading pathogens has been considered a weapon used by plants to mount a defence response (Jones & Dangl, 2006). Under stress conditions, plant cells are capable of producing a burst of ROS that is primarily composed of hydrogen peroxide (H$_2$O$_2$) (O’Brien et al., 2012). H$_2$O$_2$ triggers DNA damage, metal loss, and enzyme inactivation in iron–sulphur clusters and iron-containing proteins, as well as oxidation of cysteine thiols in a variety of proteins and small molecules (Imlay, 2013). The genus *Xanthomonas* is an important and ubiquitous group of gram-negative plant-pathogenic bacteria. Members of the genus *Xanthomonas* infect approximately 124 monocotyledonous and 268 dicotyledonous plant species and are of economic importance in regions with warm and humid climates (Böttner & Bonas, 2010).

One of the mechanisms underlying *Xanthomonas* resistance to ROS involves antioxidant enzymes, including catalases (KatA and KatG) and alkylhydroperoxide-NADPH oxidoreductase subunit C (AhpC) (Heo et al., 2010; Jittawuttipoka et al., 2009; Mongkolsum et al., 1997). In bacteria, antioxidant enzyme gene expression is controlled by the conserved redox-sensing transcription factor OxyR (Jo et al., 2015; Liu et al., 2016; Wei et al., 2012), which is activated under oxidizing conditions by the formation of a disulphide bond between two cysteine residues (Cys199 and Cys208) (Hausladen et al., 1996; Pomposiello & Demple, 2001). When activated, OxyR regulates the expression of genes involved in detoxification by binding to their promoter regions, thus activating cellular responses to H$_2$O$_2$ (Zheng & Storz, 1998). Interestingly, as a LysR-type transcriptional regulator, OxyR also inhibits its own expression during normal growth and on oxidative stress (Christman et al., 1989; Tao et al., 1991; Toledano et al., 1994). However, researchers have not clearly determined whether plants use secondary metabolites or other strategies to inhibit the transcriptional activity of OxyR or bacterial oxidative stress adaptation.

In this study, we first found that SFN weakens the ability of *Xanthomonas* to adapt to oxidative stress, thereby decreasing its virulence. Next, we showed that SFN directly binds the redox-sensing transcription factor OxyR and alters the ability of OxyR to bind to the promoters of antioxidant enzyme genes, thereby limiting the expression of these genes. Thus, our results reveal a previously uncharacterized function of the secondary metabolite SFN, which will facilitate future studies on the use of SFN as a biopesticide to control *Xanthomonas*.

## RESULTS

### 2.1 | *Xanthomonas campestris* pv. *campestris* is more resistant to SFN than *X. oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola* (Xoc)

We identified SFN as a novel plant defence priming compound that directly inhibits the growth of some bacterial and fungal pathogens when investigating the secondary metabolites directly responsible for the cessation of pathogen growth in plants (Fan et al., 2011; Schillheim et al., 2018), but we did not clearly determine whether SFN exerted the same effect on *Xanthomonas*. We investigated the potential biological effect of SFN on *Xanthomonas* species by measuring the growth of *X. oryzae* pv. *oryzae* (Xoo) PXO99A, *X. oryzae* pv. *oryzicola* (Xoc) RS105, and *X. campestris* pv. *campestris* (Xcc) XC1 in NYG medium supplemented with various concentrations of SFN. We found that 100µM SFN nearly completely abolished the growth of Xoo PXO99A (Figure 1a) and Xoc RS105 (Figure 1b), while Xcc XC1 still grew under the same conditions (Figure 1c). In addition, we tested the half-maximal effective concentration (EC$_{50}$) of SFN that...
inhibited *Xanthomonas* species growth in NYG medium. We found that the EC$_{50}$ of SFN for inhibiting the growth of Xoo PXO99A was 9.59 μM (Figure 1d) and for Xoc RS105 was 26.59 μM (Figure 1e). The value for Xcc XC1 was 225.4 μM (Figure 1f), which was approximately 10-fold higher than the values for Xoo PXO99A and Xoc RS105. Taken together, SFN inhibits the growth of *Xanthomonas* species, and Xcc is more SFN resistant than Xoo and Xoc.

2.2 | Xcc contains conserved sax gene clusters that enhance SFN resistance

We analysed the genomes of Xcc, Xoo, and Xoc using BLAST to determine why the SFN resistance of Xcc was stronger than that of Xoo and Xoc (Altschul et al., 1997). We identified four conserved sax gene clusters in Xcc XC1 (Figure 2a); these genes have been reported to be present in Psm ES4326 and Pst DC3000 and are required for the survival of Pst DC3000 in an SFN-containing environment (Fan et al., 2011). However, the Xoo PXO99A and Xoc RS105 genomes did not contain sax gene clusters. Treatments with SFN induced saxB and saxF (XCC2363 and XCC1440) expression (Figure 2b) in Xcc XC1 cells. We subsequently knocked out saxB and saxF in Xcc XC1 through a homologous recombination-based method and found that the SFN resistance of ΔsaxB/F was reduced compared with that of wild-type Xcc XC1 (Figure 2c). SFN (100 μM) nearly completely abolished the growth of ΔsaxB/F (Figure S1a). The EC$_{50}$ of SFN for inhibiting the growth of ΔsaxB/F was 25.43 μM (Figure S1b). These findings indicate that mutation of the sax gene clusters decreases the SFN resistance of Xcc.

2.3 | Mutation of saxB/F weakens the virulence and antioxidant enzyme gene expression of Xcc in planta

SFN was identified as a novel plant defence priming compound (Andersson et al., 2015; Nowicki et al., 2021), and it is present at high levels in cruciferous plants (Tilaar et al., 2012). Xcc is the causal agent of black rot disease in cruciferous vegetables (Li et al., 2020). We asked whether the virulence of Xcc is inhibited by SFN. A
leaf-clipping virulence assay using a susceptible cabbage variety (*Brassica oleracea* ‘Jingfeng No. 1’) was conducted between the wild-type strain XC1 and the SFN-sensitive strain ΔsaxB/F to answer this question. The average length of the lesions caused by the wild-type strain XC1 on cabbage leaves (1.98 cm) was significantly greater than that caused by the ΔsaxB/F strain (1.61 cm) (Figure 3a,b) at 10 days.
after inoculation. According to previous studies SFN plays an important role in maintaining the local defence response (Andersson et al., 2015), and ROS accumulation is the underlying mechanism (Zhang et al., 2018). 3,3′-diaminobenzidine (DAB) staining was performed on cabbage leaves infiltrated with buffer, XC1, or ΔsaxB/F to test the local defence response, and we found that ΔsaxB/F induced
higher levels of ROS accumulation at the infection site than buffer or XC1 (Figures 3c and S2). Next, we wanted to determine whether SFN inhibited the antioxidant activity of Xcc and affected its virulence. Consistent with our hypothesis, gene expression analysis between wild-type XC1 and ΔsaxB/F revealed that the mutation of the sax genes resulted in reduced expression of antioxidant enzyme genes (katA, katG, and ahpC) in cabbage leaves compared to XC1 (Figure 3d). Based on these results, SFN inhibits the virulence and antioxidant enzyme gene expression of Xcc in planta.

2.4 | SFN inhibits antioxidant enzyme gene expression but induces the expression of the redox-sensing transcription factor oxyR in vitro

Because the saxB/F mutant reduced the antioxidant enzyme gene expression of Xcc in planta, we next sought to determine whether SFN inhibited the expression of these genes in vitro. We cultured the XC1 strain in NYG medium to an optical density at 600 nm (OD600) of 1.0 in a 28°C shaker, and then we divided the culture into two parts: one part was supplemented with 100 μM SFN (approximately half of the EC50) and the other part was supplemented with the same volume of dimethyl sulphoxide (DMSO) as a control. After 6 h of incubation, we divided each part into two further parts: one part was treated with 1 mM H2O2 and the other part was treated with the same volume of water as a control. After 10 min of incubation, we collected all bacterial samples. The reverse transcription-quantitative PCR (RT-qPCR) analysis showed that SFN clearly inhibited the expression of antioxidant enzyme genes in XC1 grown in NYG medium (Figure 4a). Under similar test conditions, the expression of antioxidant enzyme genes in ΔsaxB/F was also inhibited by SFN (Figure S3a). Antioxidant enzyme gene expression is regulated by the redox-sensing transcription factor OxyR (Jittawuttipoka et al., 2009). We therefore explored whether SFN inhibited the expression of oxyR. For this purpose, we measured the oxyR expression level in XC1 and ΔsaxB/F after SFN induction. Surprisingly, oxyR expression was clearly increased after SFN exposure in XC1 and ΔsaxB/F (Figures 4b and S3b). Taken together, SFN inhibits antioxidant enzyme gene expression in Xcc growing in medium, but expression of the redox-sensing transcription factor oxyR is induced under the same conditions.

2.5 | SFN inhibits the H2O2 resistance of Xcc

ROS are a key feature of the plant defence against invading pathogens (Fones & Preston, 2012). As a result, plant pathogens must be able to either prevent ROS production or tolerate high concentrations of these highly reactive chemicals, and antioxidant enzymes play an important role in helping bacterial pathogens tolerate ROS resistance (Heo et al., 2010). SFN inhibits the expression of antioxidant enzyme genes, thereby influencing the ability of Xcc to adapt to oxidative stress. Next, we tested the resistance of Xcc to H2O2, one of the most important ROS. Diffusion-based H2O2 resistance assays were conducted in which exponentially growing cells were spread on NYG agar plates that contained various concentrations of SFN. Then, a hole was punched in the centre of each plate and

![Figure 4](https://example.com/figure4.png)

**FIGURE 4** Analysis of antioxidant gene expression in sulforaphane (SFN)-treated Xanthomonas campestris pv. campestris XC1 cells. (a) SFN inhibited XC1 antioxidant gene expression in vitro. XC1 cells were incubated with 100 μM SFN for 6 h and then the cultures were treated with 1 mM H2O2 for 10 min, after which samples were collected for the reverse transcription-quantitative PCR (RT-qPCR) assay. (b) SFN induced oxyR expression in XC1 in vitro. XC1 cells were incubated with 20 μM SFN for 6 h and oxyR transcript levels were determined using RT-qPCR. 16S rRNA was used as the endogenous control, the expression level in the wild-type strain was assigned a numerical value of 1, and transcript levels were normalized to the 16S rRNA level. The bars indicate the mean ± SD (n = 3) (a and b). Experiments were performed three times with similar results. The statistical analysis was performed using two-way analysis of variance followed by Tukey’s multiple comparisons test (**p < 0.001, *p < 0.05; n = 3).
20 μl of H2O2 (60 mM) was pipetted into the hole. After 2 days of incubation, the inhibitory zone diameters of the cultures were measured and compared. We found that 100 μM SFN decreased the H2O2 resistance of XC1, and the phenomenon was more obvious in ΔsaxB/F (Figure 5). Even 20 μM SFN clearly inhibited the adaptation of ΔsaxB/F to oxidative stress. Nevertheless, the H2O2 resistance of the saxB/F mutant was not different from that of XC1 in the absence of SFN (Figure 5b). These data confirm that SFN inhibits the adaptation of Xcc to oxidative stress.

2.6 SFN directly targets OxyR to inhibit the Xcc adaptation to oxidative stress

The OxyR transcriptional regulator of most bacterial phytopathogens activates the expression of defence genes in response to oxidative stress (Mongkolsuk et al., 1997; Toledano et al., 1994; Wei et al., 2012). Our findings showed that SFN induced oxyR expression in vitro (Figure 4b). An oxyR gene deletion mutant (ΔoxyR) was constructed to test the role of OxyR in the response to SFN. On testing the H2O2 resistance of XC1 and ΔoxyR treated with or without SFN, we found that the H2O2 resistance of ΔoxyR was clearly reduced compared with that of XC1 and SFN did not alter the H2O2 resistance of the ΔoxyR mutant compared with that of XC1 (Figure 6a,b). In addition, using RT-qPCR, we verified the results of a previous report showing that loss of oxyR inhibited antioxidant enzyme gene expression (Figure 6c). We purified tagged OxyR-His protein (Figure S4) and performed a microscale thermophoresis (MST) assay to further examine whether SFN inhibited H2O2 resistance in XC1 cells through an OxyR-mediated mechanism. The MST assay verified direct binding between the OxyR protein and SFN (Kd = 0.3254 ± 0.1046 μM) (Figure 6d). In this experiment, we used the DNA-directed RNA polymerase subunit α (RpoA)-GST fusion as a negative control, and phosphate-buffered saline (PBS) was used as a blank control to show the specific binding between the OxyR protein and SFN. These results suggest that SFN directly targets OxyR to inhibit the H2O2 resistance of XC1.

2.7 SFN inhibits the ability of OxyR to bind to the promoters of antioxidant enzyme genes

OxyR directly binds to the promoter regions of target genes to directly regulate their expression (Jittawuttipoka et al., 2009;
We performed an electrophoretic mobility shift assay (EMSA) to test whether direct binding between SFN and the OxyR protein altered the ability of the OxyR protein to bind to the promoter of antioxidant enzyme genes. First, we cloned the putative promoter DNA fragments that covered approximately 500 bp upstream of the Xcc katG and Xcc katA translational start sites, namely, p-katG and p-katA. The addition of purified OxyR-His protein (at

![Figure 6](image-url)

**Figure 6** Identification of OxyR as a target of sulforaphane (SFN) that mediates the H₂O₂ resistance of *Xanthomonas campestris* pv. *campestris* XC1. (a and b) OxyR is responsible for the SFN-mediated H₂O₂ sensitivity of XC1. The H₂O₂ (60 mM) resistance of XC1 and ΔoxyR in SFN (100 μM)-containing medium was examined by performing a diffusion assay. Experiments were performed three times with similar results. Each dot represents the inhibitory zone diameter of a single biological replicate (n = 10). ***p < 0.001 (two-way analysis of variance [ANOVA] followed by Tukey’s multiple comparisons test). (c) OxyR regulates antioxidative gene expression in XC1. XC1 and ΔoxyR were grown in NYG medium to an OD₆₀₀ of 1.0 then 1 mM H₂O₂ was added to the medium and the cells were incubated for 10 min. The expression of antioxidant genes (katG, katA, and ahpC) in XC1 and ΔoxyR was examined using reverse transcription-quantitative PCR. The bars represent the mean ± SD (n = 3). 16S rRNA was used as the endogenous control, the expression level in the wild-type strain was assigned a numerical value of 1 and transcript levels were normalized to the 16S rRNA level. Experiments were performed three times with similar results. The statistical analysis was performed using two-way ANOVA followed by Tukey’s multiple comparison test (***p < 0.001). (d) SFN directly binds to OxyR. Microscale thermophoresis (MST) was used to quantify the binding affinity between SFN and the OxyR protein. Proteins were incubated with SFN in label-free standard capillaries to conduct the MST assay. The titres of SFN ranged from 0.0305 to 1000 μM. The RpoA-GST fusion protein was used as a negative control and phosphate-buffered saline (PBS) was used as a blank control. The solid curve shows the fit of the data points to the standard Kᵩ fit function, Kᵩ, dissociation constant.
concentrations ranging from 0 to 1 μM) to the reaction mixtures (20 μl, 25°C, 10 min) caused a shift in the mobility of the p-katG and p-katA DNA fragments, suggesting that under these conditions the OxyR-His protein directly bound p-katG and p-katA (Figure 7a,b). However, the shift induced by the formation of the complex of the OxyR-His protein with p-katG or p-katA was inhibited by the addition of SFN: as the concentration of SFN increased from 0 to 125 μM, the signals of the shifted binding complex bands became increasingly weaker, while the signals of the free probe bands became increasingly stronger (Figure 7). We used the saxF promoter as a negative control: the saxF promoter did not bind to the OxyR protein under SFN-containing or SFN-free conditions. Overall, our findings are the first to show that the molecular mechanism underlying the decreased antioxidant enzyme gene expression induced under oxidative stress conditions is the SFN-mediated inhibition of OxyR DNA-binding ability.

2.8 | SFN inhibits the \( \text{H}_2\text{O}_2 \) resistance of Xoc and Xoo

A sequence analysis of the oxyR gene revealed that OxyR is a conserved redox-sensing transcription factor in *Escherichia coli*, *Pseudomonas*, and *Xanthomonas* (Figure S5). We measured oxyR expression in Xoo PXO99A and Xoc RS105 grown in NYG medium containing 20 μM SFN to further investigate whether SFN alters the oxidative stress adaption ability of other *Xanthomonas* species. Using the RT-qPCR assay, we found that SFN induced oxyR expression in Xoo PXO99A and Xoc RS105 (Figure 8a,b). In addition, we tested the \( \text{H}_2\text{O}_2 \) resistance of Xoo PXO99A and Xoc RS105 in the presence of SFN, as described above. Notably, 20 μM SFN clearly reduced the \( \text{H}_2\text{O}_2 \) tolerance of Xoo PXO99A (Figure 8c) and Xoc RS105 (Figure 8d). We also detected antioxidant enzyme gene expression in Xoo PXO99A and Xoc RS105 after SFN induction. As shown in Figure S6, SFN clearly inhibited the expression of antioxidant enzyme genes in Xoo and Xoc.
FIGURE 8  Sulforaphane (SFN) inhibits the H$_2$O$_2$ resistance of *Xanthomonas oryzae* pv. *oryzae* (Xoo) PX099A and *X. oryzae* pv. *oryzicola* (Xoc) RS105 growing in medium. (a and b) SFN induces oxyR expression in Xoc and Xoo. Xoo and Xoc cells were incubated with 20mM SFN for 6 h in induction medium and transcription levels of the indicated genes were determined using reverse transcription-quantitative PCR. 16S rRNA was used as the endogenous control, the expression level in the wild type was assigned a numerical value of 1, and transcript levels were normalized to the 16S rRNA level. Experiments were performed three times with similar results. The bars represent the mean ± SD (n = 3). (c and d) SFN inhibits the H$_2$O$_2$ resistance of Xoo PXO99A and Xoc RS105. H$_2$O$_2$ tolerance was determined by performing diffusion assays in which exponentially growing cells were spread on NYG agar plates containing SFN at different concentrations and 20μl of H$_2$O$_2$ (30mM) in a central hole. Experiments were performed three times with similar results. Each dot represents the inhibitory zone diameter of a single biological replicate (n = 5). ***p < 0.001, **p < 0.05 (unpaired t test).
Based on these findings, SFN inhibits the oxidative stress adaptation ability of Xoo PXO99A, Xoc RS105, and Xcc XC1.

3 | DISCUSSION

Secondary metabolites have long been suggested to contribute to the interactions of plants with other organisms (Hartmann, 2008), especially in plant responses to microbial pathogens, which are among the most extensively studied plant immune responses (Piasecka et al., 2015). Nevertheless, the precise mechanisms underlying the contribution of secondary metabolites to plant immunity have rarely been studied. In the present study, we found that SFN, a secondary metabolite of crucifers, inhibited not only the virulence and adaptative oxidative stress ability of the crucifer pathogen Xcc but also the oxidative stress adaptation ability of the crucifer nonpathogens Xoo and Xoc. In addition, we also dissected the mechanism by which SFN inhibits the oxidative stress adaptation ability of Xcc. The antioxidative stress transcription factor OxyR is a target of SFN in Xcc. Direct binding between SFN and OxyR weakens the ability of OxyR to bind to the promoters of antioxidant enzyme genes, leading to the inhibition of antioxidant enzyme gene expression under oxidative stress conditions (Figure 9). Our findings collectively revealed a novel function of SFN in the regulation of the oxidative stress adaptation ability of Xanthomonas species.

SFN is well known for its dual role in helping cruciferous plants defend themselves against pathogens: SFN not only directly inhibits pathogen growth (Fan et al., 2011) but also functions as a defence-priming compound in the local plant defence response (Schillheim et al., 2018). In addition, SFN also plays an important role in mediating the nonhost resistance of Arabidopsis (Fan et al., 2011), and it inhibits the growth of nonhost Pseudomonas species in Arabidopsis plants. In the current study, we found that SFN inhibited the growth of Xcc, Xoc, and Xoo at different concentrations. Interestingly, due to the presence of sax genes in Xcc, this species exhibited stronger SFN resistance than Xoc and Xoo (Figure 2). When Xcc was grown in an SFN-containing environment, the expression of the sax genes was clearly induced (Figure 2b). Our results support the findings of previous reports showing that sax genes play an important role in mediating SFN resistance in bacteria. However, the function of sax in SFN resistance and the mechanisms by which bacteria sense SFN and regulate sax gene expression remain unclear. We suspect that sax gene expression is regulated by an SFN-sensing transcription factor: on sensing SFN, the transcription factor becomes activated to regulate sax gene expression. This hypothesis is being tested in our laboratory using a combination of surface plasmon resonance (SPR)
and RNA-sequencing (RNA-Seq) approaches, and the results will be reported in a separate article in the future.

To date, the mechanism by which SFN directly inhibits the growth of pathogens in planta has not been elucidated. In the present study, we found that in addition to participating in regulating Xcc growth, SFN also modulated virulence and oxidative adaptation ability (Figure 3). Plant innate immune responses to bacterial infection include an oxidative burst via increased levels of ROS (Fones & Preston, 2012), which are toxic to bacterial cells and cause damage to proteins, nucleic acids, and cell membranes (Cabasil et al., 2000). Under oxidative stress, bacteria employ multiple scavenging enzymes (catalases and peroxidases) to degrade H₂O₂ (Liu et al., 2016). The expression of scavenging enzymes in response to oxidative stress is regulated by peroxide-sensing transcription factors and the peroxide-sensing regulator OxyR has been the most extensively studied of these factors in many bacteria (Hahn et al., 2002; Liu et al., 2016; Mongkolsuk et al., 2000). However, researchers have not determined whether the transcriptional activity of OxyR is regulated by plants. Here, we found that the secondary metabolite SFN directly binds to OxyR and inhibits its transcriptional regulatory activity. These findings showed for the first time that when plants are invaded by pathogens, they not only produce a burst of ROS to kill invaders, but also synthesize secondary metabolites to inhibit the antioxidant ability of invaders. OxyR is a LysR-type transcriptional regulator; during normal growth and under oxidative stress, OxyR inhibits its own expression (Toledano et al., 1994). Here, we found that oxyR expression was induced by SFN, but antioxidant gene expression was inhibited by SFN under oxidative stress conditions. When the transcriptional regulatory activity of OxyR, a transcriptional inhibitor for itself, was inhibited by SFN, oxyR expression increased, which might also explain why SFN induced the expression of antioxidant genes but inhibited the expression of oxyR.

The genus Xanthomonas represents a large group of gram-negative bacterial plant pathogens that cause diseases in at least 124 monocot and 268 dicot species (Hayward, 1993). Antimicrobial therapies are generally used in agriculture to prevent disease caused by Xanthomonas. However, the abuse or inappropriate use of agricultural antibiotics has led to significant antimicrobial resistance. The use of the commercially available bactericide bismuthexazol has already resulted in the emergence of bismuthexazol-resistant strains of Xoo. Antimicrobial resistance to agricultural antibiotics has led to the prioritization of the search for antibiotics with new modes of action. In our study, SFN effectively inhibited the growth of Xoo and Xoc in NYG medium at relatively low concentrations \( EC_{50} = 9.59 \mu M \) for Xoo and \( EC_{50} = 26.59 \mu M \) for Xoc. In addition, SFN is produced by plants and it is also used as a medicine to kill human cancer cells (Ramirez & Singletary, 2009), therefore it is environmentally friendly and can be used in humans. Overall, SFN may be useful as an environmentally friendly antibiotic to control Xanthomonas pathogens in agriculture.

In summary, in the present study we identified a novel function of SFN in helping plants defend themselves against invading Xanthomonas pathogens. In addition, we identified the SFN target OxyR and we dissected the mechanism by which SFN inhibits the ability of Xanthomonas to adapt to oxidative stress. This novel function might also help us understand why SFN mediates the nonhost resistance of Arabidopsis. Our research will assist future studies examining the use of SFN as an antibiotic to control Xanthomonas species.

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 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), and rifampicin (Rif, 50 μg/ml) were added to the growth medium for selection.

4.2 | Growth assays

Xanthomonas strains were grown overnight in NYG medium at 28°C with shaking at 200 rpm. The optical densities of the cultures were adjusted to an OD₆₀₀ of 1.0 and the cultures were diluted 1:100 in 25 ml of fresh NYG medium containing different concentrations (0, 20, 40, 100 μM) of SFN. Growth curves were monitored by measuring the OD₆₀₀ every 4 h after inoculation and all inoculated samples were grown at 28°C until the stationary phase was achieved. Three biologically independent experiments were performed.

4.3 | Plant material and bacterial virulence assays

The plant material and bacterial virulence assays have been described previously (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 about 70% relative humidity. Plants were inoculated at 6 weeks with bacterial suspensions at an approximate OD₆₀₀ of 0.1 in sterile distilled water by immersing 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.

4.4 | Deletion of the Xcc oxyR gene

Generation of the in-frame deletion mutant was conducted using wild-type Xcc XC1 as the parental strain via allelic homologous recombination (Li et al., 2020; Wang et al., 2018). The 500-bp DNA fragments flanking the Xcc oxyR gene were amplified with Pfu DNA polymerase using Xcc XC1 genomic DNA as a template and...
the corresponding primer pairs (Table S2). Fragments were purified and ligated into pK18mob sacB with the SE Seamless Cloning and Assembly Kit (ZOMANBIO) to obtain the plasmid pK18-oxyR. The resulting construct was transferred into Xcc 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 Xcc oxyR deletion strain (ΔoxyR) was obtained.

### 4.5 Protein expression and purification

Protein expression and purification were performed as described previously (Li et al., 2020; Wang et al., 2018). Briefly, the coding regions of oxyR (Xcc0832) and rpoA (Xcc0919) were amplified by PCR using the corresponding primer pairs (Table S2). The PCR products were purified, digested, and cloned into pET30a or pGEX-6p-1, creating the final constructs pET30a-oxyR and pGEX-6p-1-rpoA, respectively. These vectors were transformed into E. coli BL21 (DE3) for protein expression. Briefly, the transformed strain was cultivated in LB medium at 100 μg/ml Amp or 50 μg/ml Km overnight at 37°C. Then, a 5 ml overnight culture was transferred into 500 ml of fresh LB medium containing 100 μg/ml Amp or 50 μg/ml Km overnight at 37°C. Then, a 5 ml overnight culture was transferred into 500 ml of fresh LB medium containing 100 μg/ml Amp or 50 μg/ml Km and grown at 37°C with shaking at 220 rpm until OD600 = 0.4. Subsequently, isopropyl β-D-1-thiogalactopyranoside (IPTG) 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 x g) at 4°C and resuspended in 15 ml of PBS supplemented with phenyl methyl sulfonyl fluoride (PMSF) 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 x g and 4°C. For OxyR-His protein purification, soluble protein fractions were collected and mixed with Ni-NTA agarose (Sigma–Aldrich) for 2 h at 4°C; the mixture was then placed into a column and extensively washed with PBS containing 40 mM imidazole. The proteins were subsequently eluted using elution buffer containing 300 mM imidazole. For RpoA-GST protein purification, 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 sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), and the protein concentration was determined using a Bradford protein assay kit (Bio-Rad).

### 4.6 Electrophoretic mobility shift assay

EMSA s were performed as described previously (Heo et al., 2010). DNA fragments of the katA, katG, and saxF promoter regions were generated by PCR using the corresponding primer pairs (Table S2). The probe DNA (20 ng) was incubated with purified His-tagged OxyR in 20 μl of binding buffer (2 mM Tris–HCl [pH 7.8], 0.1 mM EDTA, 0.2 mM dithiothreitol [DTT], 4 mM KCl, 0.5 mM MgCl2, 10 ng/ml bovine serum albumin [BSA], and 10% glycerol) containing 1 μg of poly(dI-dC) for 10 min at 25°C. The DNA–protein mixture was resolved on a 5% native polyacrylamide gel in 0.5 x Tris-borate-EDTA buffer at 4°C. The gel was soaked in 10,000-fold-diluted SYBR Green I nucleic acid dye (Sangon Biotech) and the DNA was visualized at 300 nm.

### 4.7 RT-qPCR assay

The transcription of target genes was detected using RT-qPCR as described previously (Wang et al., 2018). Bacterial cells were collected when the cellular OD600 reached 1.0 in NYG medium with or without stress. The samples were then collected to isolate total RNA using 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. DNA 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 analysis was performed using the 2−ΔΔCt method with 16S rRNA serving as the endogenous control and the expression level in the wild-type strain was assigned a numerical value of 1. The experiments were performed three times, each involving three replicates.

### 4.8 Analysis of antioxidant enzyme gene expression

Xcc XC1 and ΔsaxB/F were grown in NYG medium containing 50 mg/L Rif at 28°C to an OD600 of 1.0 to measure antioxidant enzyme gene expression in the presence of H2O2 and SFN. Then, SFN was added to the bacterial medium at different concentrations and the cells were incubated for 6 h at 28°C (Wang et al., 2021). Next, the cultures were treated with 1 mM H2O2 for 10 min, after which the samples were extracted. Finally, antioxidant enzyme gene (katA, katG, and ahpC) expression was detected using RT-qPCR as described above. The primers used for RT-qPCR are listed in Table S2.

*Xanthomonas* cells were cultured overnight, harvested, washed twice with sterile water, and resuspended in sterile water to a final OD600 of 1.0 to determine the transcript levels of antioxidant enzyme genes in planta. Fully expanded leaves of 6-week-old plants were infected with bacterial suspensions by clipping and harvested.
96 h later. Total RNA was extracted using the Eastep Super Total RNA Extraction Kit (Promega) and DNA was removed with RNase-free DNase (Promega). RT-qPCR was performed as described above. One hundred nanograms of total RNA were used to amplify selected bacterial genes. Transcript levels were normalized to the 16S rRNA level. The primers used for RT-qPCR are listed in Table S2.

### 4.9 | Stress tolerance assay

The stress tolerance assay was performed on agar plates as described previously with minor modifications (Wang et al., 2021). Bacterial strains were grown in NYG medium until the OD\textsubscript{600} reached 1.0 (mid-exponential phase). The mid-exponential-phase cultures were collected and suspended in fresh NYG medium at an OD\textsubscript{600} of 0.01. Then, 5 ml of each of the resulting cultures were spread onto NYG agar plates with or without SFN (various concentrations), a hole was punched in the centre of each plate, and 20 μl of H\textsubscript{2}O\textsubscript{2} (60 mM) or SFN (20 μM) was pipetted into the hole as appropriate for selection. All the plates were incubated at 28°C for 3 days, and the growth inhibition zones surrounding the holes were measured. All assays were performed at least three times.

### 4.10 | DAB staining assay

*Brassica oleracea* leaves were infiltrated with different *Xanthomonas* suspensions at an OD\textsubscript{600} of 0.1 and MgCl\textsubscript{2} (10 mM) buffer using a needleless syringe as previously described to visualize H\textsubscript{2}O\textsubscript{2} accumulation (Wu et al., 2021). After 24 hours, the infected leaves were stained with DAB (Sigma). The stained leaves were observed and photographed under an optical microscope. The DAB signal intensity was calculated from the digital photographs by determining the number of brown pixels using a previously described method (Gottig et al., 2018). Average DAB signal intensities were calculated from at least 10 photographs of differently treated leaves from three independent experiments.

### 4.11 | MST measurement

MST measurement was used for detecting the interaction between OxyR and SFN by quantifying the thermophoretic movement of fluorescent molecules in response to a temperature gradient. If the fluorescent molecule interacts with the ligand, the molecular properties of the molecules, such as charge, size, and hydration shell, will influence the molecular motility (Deng et al., 2018; Huang & Zhang, 2021). We used the RpoA-GST protein as a negative control, and we used PBS as a blank control to show the specific binding between the OxyR protein and SFN. Briefly, 10 μM purified OxyR-His and RpoA-GST were labelled with the Monolith NT Protein Labeling Kit RED-NHS (NanoTemper Technologies GMBH) using the red fluorescent dye NT-647 N-hydroxysuccinimide (amine-reactive) according to the manufacturer’s instructions. The excess labelling reagents were removed by buffer-exchange column chromatography, and the labelled OxyR-His and RpoA-GST proteins were eluted in NTA buffer (300 mM NaCl and 50 mM sodium phosphate buffer, pH 7.0). The binding assays were performed on a Monolith NT.115 MST instrument (NanoTemper Technologies GMBH) using capillaries treated in a standard manner. The \( K_d \) Fit function of NanoTemper Analysis software v. 1.5.41 was used for curve fitting and the calculation of the value of the dissociation constant (\( K_d \)).

### 4.12 | Statistical analysis

The experimental datasets were subjected to analysis of variance 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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### 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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