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Tizoxanide associated with anti-virulent activity controls fire blight disease caused by *Erwinia amylovora* in *Malus asiatica*



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Erwinia amylovora is a causative pathogen of fire blight disease, affecting apple, pear, and other rosaceous plants. This study aimed to discover new anti-virulence agents to control this plant bacterial disease by preventing its pathogenesis. To this end, 2485 small molecules were screened for targeting the type III secretion system (T3SS) which is a critical virulence factor in *E. amylovora*. Our screening identified tizoxanide as a potent inhibitor, significantly reducing the promoter activity of *hrpA*, which encodes the Hrp pilus protein essential for T3SS function. Beyond T3SS inhibition, tizoxanide also exhibited inhibitory effects on other virulence factors by down-regulating genes associated with these functions. Given its broad effects on various virulence factors, we conducted a proteomic analysis of *E. amylovora* treated with tizoxanide, and the results revealed differentially expressed proteins related to cell metabolism and pathogenesis. In a plant infection model, spray treatment with tizoxanide effectively protected Chinese pearleaf crabapple seedlings against *E. amylovora*. Furthermore, a synthesized tizoxanide derivative containing an isoxazole moiety further enhanced *hrpA* promoter inhibition and disease control efficacy compared to the tizoxanide. Taken together, our results highlight the potential of tizoxanide as an anti-virulence agent to prevent plant disease caused by *E. amylovora*.

Plant disease outbreaks significantly reduce agricultural yield and quality, leading to economic losses and concerns about global food security^{1,2}. Fire blight, caused by *Erwinia amylovora*, is an economically important plant disease affecting apple and pear production worldwide³. Since the discovery of this disease on Rosaceae plants in New York in 1780, fire blight has continued to spread to new regions⁴. The causal agent *E. amylovora* is a Gram-negative bacterial pathogen belonging to the Enterobacterales. It can infect various plant tissues, including tree trunks, shoots, flowers, and natural wounds, through abiotic and biotic vectors⁵. Symptoms of fire blight include necrotic lesions, bacterial exudate, and tree cankers, which are observed in branches, blossoms, and fruits⁶. The *E. amylovora* genome, ~3.8 Mb in size, encodes over 3400 genes, including those related to pathogenicity, such as type III secretion system (T3SS), exopolysaccharide (EPS), flagella motility, and Lon protease^{7,8}. Functional studies have demonstrated the importance of these virulence factors in *E. amylovora*. For example, deletion mutants of T3SS and amylovoran biosynthesis genes

showed a loss of pathogenicity on immature pears⁹; the genes *hrpA* and *hrpS* encoding the Hrp pilus protein and δ^{54} -dependent enhancer-binding protein, respectively, were shown to be essential for *E. amylovora* pathogenicity¹⁰.

Fire blight management has relied on antibiotics such as streptomycin, oxolinic acid, and copper-based compounds¹¹. However, the overuse of antibiotics has led to the emergence of resistant *E. amylovora* in orchards¹². To overcome this issue, alternative strategies such as biological and physical controls have been explored. Biological control agents, including bacteriophage cocktails and plant extracts, have shown promising antibacterial activity against *E. amylovora* in vitro and in vivo^{13,14}. However, their efficacy can vary depending on host species, environmental conditions (e.g., temperature and humidity), and nutritional status, limiting their reliability compared to chemical agents¹⁵. As an alternative to antibiotics, anti-virulence compounds have gained attention; these compounds selectively inhibit the virulence traits of the pathogen, such as T3SS, amylovoran

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production, bacterial biofilms, and quorum sensing, without affecting bacterial growth. This approach minimizes the development of antibiotic resistance compared to traditional bactericidal compounds¹⁶.

The T3SS is a key virulence factor widely found in Gram-negative bacteria, including plant pathogens such as *Pseudomonas*, *Xanthomonas*, and *Ralstonia*, as well as animal pathogens such as *Salmonella* and *Shigella*¹⁷. T3SS consists of effector proteins and structure components that enable bacteria to invade host organisms directly. In plant-pathogen interactions, effector proteins are delivered into plant cells by T3SS, having a crucial role in infection¹⁸. T3SS, one of the most complex bacterial nanomachines, is composed of over 20 different proteins, and its structure includes four main components: an ATPase, a basal body spanning the inner and outer membranes, a needle-like pilus, and a translocon that forms a pore in the plant membrane¹⁹. The genes encoding T3SS are located in pathogenicity islands and are clustered *hrp/hrc* genes, which are regulated by environmental factors such as nutrient availability, pH, and temperature^{6,20}. Given the importance of *E. amylovora* pathogenesis, several studies have demonstrated the efficacy of T3SS inhibitors in reducing *E. amylovora* pathogenicity, making them a promising agent for fire blight management^{21,22}. While significant efforts have been made to identify T3SS inhibitors for improved bacterial disease control, the discovery of novel anti-virulence compounds remains essential. In addition, further research is needed to evaluate their potential applications in agriculture.

This study aimed to identify anti-virulence compounds to mitigate the pathogenicity of *E. amylovora* and develop effective fire blight management strategies. To do this, a total of 2485 small molecules were screened using a green fluorescent protein (GFP) reporter assay driven by the *hrpA* promoter. The identified compound effectively modulated T3SS and associated

virulence factors in vitro. To further understand the biological and chemical functions of the identified compound, we performed a comparative proteome analysis of *E. amylovora* and synthesized derivatives to enhance T3SS inhibition activity. Given the promising disease control efficacy of the identified compound against fire blight, our findings provide a strong foundation for developing anti-virulence therapeutic strategies to manage bacterial diseases in agriculture.

Results and discussion

Discovery of small molecules as a T3SS inhibitor against *E. amylovora*

To find small molecules that inhibit T3SS expression of *E. amylovora*, we generated a transgenic strain of *E. amylovora* containing the *hrpA* promoter fused to a promoterless GFP vector, designated as the HK0955 strain (Supplementary Fig. 1). An initial screening of 2485 compounds from a clinical chemical library (CCL) was conducted, each tested at a concentration of 25 μ M, to evaluate their ability to inhibit GFP expression driven by the *hrpA* promoter (Fig. 1a, b). As a result, 57 compounds exhibited reduced GFP expression by more than 50% compared to the dimethyl sulfoxide (DMSO) control (Fig. 1a, b). Further analysis of these 57 compounds using 2-fold serial dilutions, starting with a concentration of 100 μ g/mL, revealed that 9 compounds inhibited GFP expression by over 80% at a concentration of 100 μ g/mL (Fig. 1c): CCL1 (piroctone olamine), CCL2 (nitazoxanide), CCL3 (ciclopirox olamine), CCL4 (benzethonium chloride), CCL5 (triclosan), CCL7 (epetaborole hydrochloride), CCL8 (tizoxanide), CCL9 (tetrachlorocatechol), and CCL10 (4-chloromethyl-6,7-dihydroxy-chromen-2-one) (Supplementary Fig. 2). In addition, these nine compounds inhibited GFP expression by over 50% at a concentration of 25 μ g/mL. Regarding

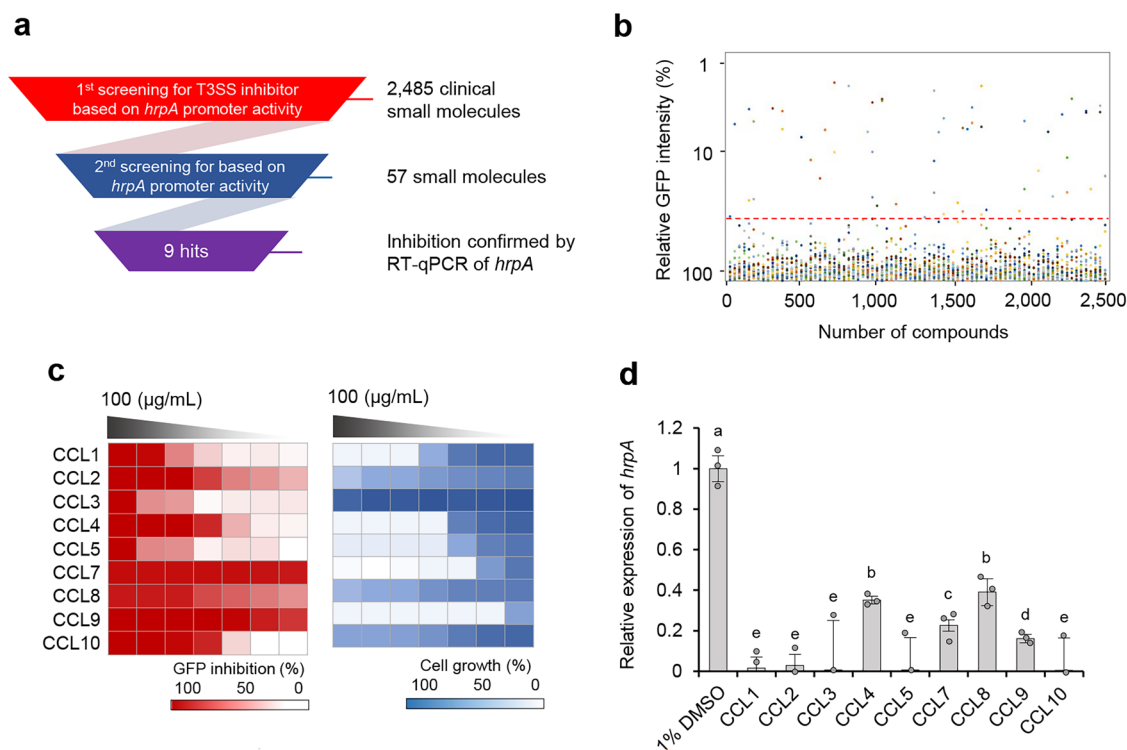


Fig. 1 | Chemical library screening for the discovery of T3SS inhibitors.

a Schematic workflow for the screening of chemical compounds targeting the T3SS. **b** Primary screening result of 2485 small molecules based on the relative GFP intensity controlled by the *hrpA* promoter. GFP inhibition was investigated in *E. amylovora* HK0955 grown in HMM medium supplemented with the small molecules (25 μ M) at 18 $^{\circ}$ C for 18 h. Compounds above the red dot line exhibited reduced GFP expression by more than 50%. **c** Effects of nine hit compounds on the *hrpA* promoter activity and bacterial growth. Effects of the nine hit compounds on GFP inhibition and bacterial growth. For the *hrpA* promoter activity assay, each

compound was serially two-fold diluted, starting at a concentration of 100 μ g/mL in HMM medium containing bacterial cells (2×10^8 CFU/mL). For the antibacterial activity assay, each compound was serially two-fold diluted, starting at a concentration of 100 μ g/mL in MGY medium containing bacterial cells (1×10^5 CFU/mL). **d** Transcript levels of the *hrpA* gene of *E. amylovora* TS3128 treated with each hit compound at a concentration of 25 μ g/mL. Total RNA was isolated from *E. amylovora* TS3128 grown in HMM medium supplemented with the hit-compounds for 18 h. Different letters above each bar represent the significant difference based on ANOVA with Tukey's HSD test ($P < 0.05$).

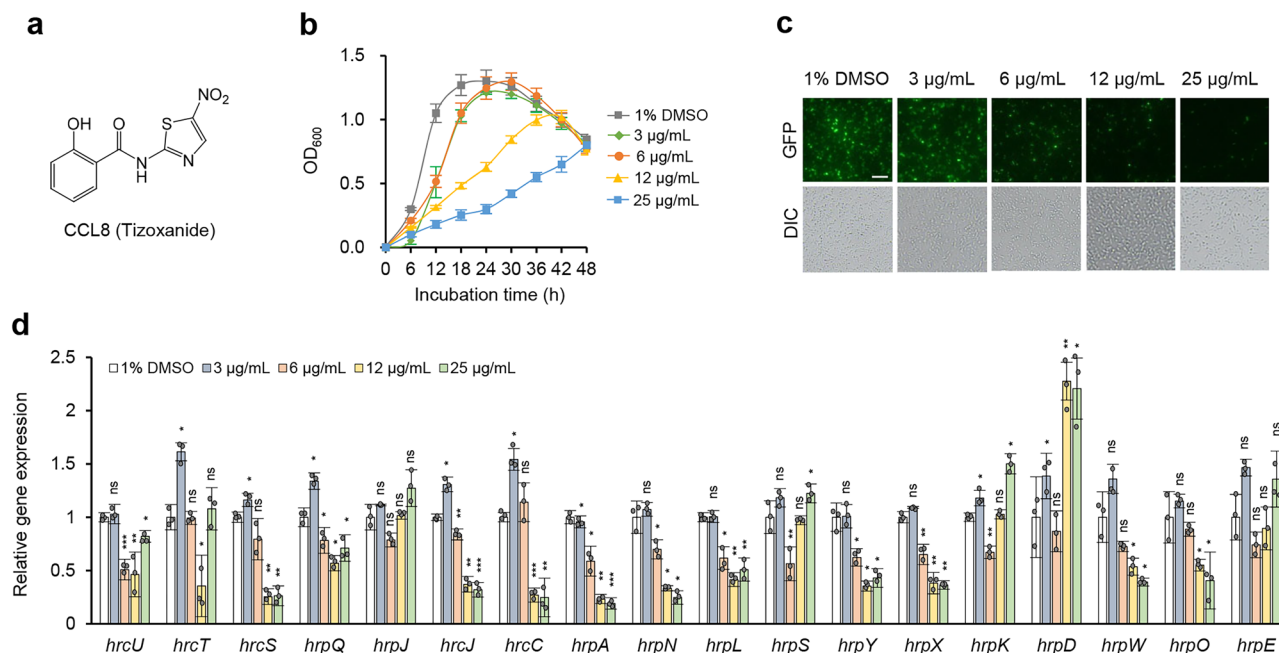


Fig. 2 | Effects of tizoxanide on bacterial growth and T3SS gene expression.

a Chemical structure of tizoxanide. **b** Growth of *E. amylovora* TS3128 in LB medium at 28 °C following treatment with various concentrations of tizoxanide. **c** Effects of tizoxanide on the *hrpA* promoter activity. Images were captured 18 h after treatment with tizoxanide. Scale bar, 40 µm. **d** Relative expression of T3SS-related genes in *E.*

amylovora TS3128 treated with different concentrations of tizoxanide. Total RNA was isolated from the *E. amylovora* TS3128 grown in HMM medium supplemented with tizoxanide for 18 h. ns, not significantly different; *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$ based on Student's *t*-test.

bactericidal activity, four small molecules CCL2, CCL3, CCL8, and CCL10 exhibited no bactericidal activity at a concentration of above 25 µg/mL, and the other molecules CCL1, CCL4, CCL5, CCL7, and CCL9 exhibited an antibacterial activity at a concentration of 25 µg/mL. Notably, the four compounds CCL2, CCL3, CCL8, and CCL10 inhibited the *hrpA* promoter activity without any bactericidal activity at a concentration above 25 µg/mL (Fig. 1c). Furthermore, when we examined the *hrpA* gene expression in *E. amylovora* treated with each compound at a concentration of 25 µg/mL, all the tested compounds reduced the *hrpA* expression by more than 50% (Fig. 1d). Taken together, among the nine hit compounds, we determined that compounds CCL2, CCL3, CCL8, and CCL10 affect the *hrpA* promoter activity without any significant antibacterial activity; among these four compounds, CCL2 (nitazoxanide) and CCL8 (tizoxanide) are structurally similar (Supplementary Fig. 2). Considering that nitazoxanide is rapidly hydrolyzed in vivo into its active metabolite tizoxanide²³, and based on our observation that tizoxanide exhibited better *hrpA* promoter inhibitory activity at lower concentrations than nitazoxanide (Fig. 1c), we decided to further investigate the biological and chemical functions of the tizoxanide, focusing on the pathogenicity of *E. amylovora*.

Tizoxanide inhibited the expression of T3SS genes with no bactericidal activity

Tizoxanide belongs to the thiazolidine class which constitutes a thiazole ring with a nitro group and phenol with a hydroxyl group (Fig. 2a). Tizoxanide, also known as a desacetyl-nitazoxanide, is a metabolite of nitazoxanide formed in living organisms through hydrolysis²³. It has been reported that nitazoxanide inhibits pyruvate:ferredoxin/flavodoxin oxidoreductases against parasites and anaerobic bacteria and was weakly active against the pyruvate dehydrogenases (PDHs) of *E. coli*²⁴. When the PDH activity of *E. amylovora* was measured in the presence of tizoxanide, the PDH activity was reduced by 25% and 44% at concentrations of 6 and 25 µg/mL, respectively (Supplementary Fig. 3).

When the growth rate of *E. amylovora* in LB medium supplemented with tizoxanide was measured, tizoxanide did not show any bactericidal activity at the tested concentrations, but it exhibited growth retardation of

E. amylovora at concentrations of 12 and 25 µg/mL (Fig. 2b). However, tizoxanide effectively inhibited GFP expression controlled by the *hrpA* promoter at concentrations of 6 µg/mL or higher (Fig. 2c). When the transcriptional levels of 18 T3SS related genes in *hrp/hrc* gene clusters were quantified, 12 T3SS related genes were significantly down-regulated at concentrations of 6 µg/mL or higher by the tizoxanide treatment, but 6 genes including *hrpS*, *hrpK*, and *hrpD* were not suppressed by tizoxanide at a concentration of 25 µg/mL (Fig. 2d).

The gene *hrpL* is known as the master regulator that recognizes and binds to specific sequences (*hrp*-box) on T3SS genes²⁴. The expression of *hrpL* is negatively or positively controlled by other regulators, including GrrS/GrrA, EnvZ/OmpR, HrpS, HrpX/Y, and CsrA⁸. Among these regulators, a bacterial enhancer-binding protein HrpS interacts with IHFaβ, YhbH, and RNA polymerase and induces *hrpL* expression in *E. amylovora*¹⁰. As a transcriptional regulator, *hrpL* directly or indirectly governs the expression of effector proteins, such as DspA, Eop1, Eop3, and AvrRpt2, that have critical roles in modulating host cellular processes to benefit bacterial infection²⁵. Given that *hrpL* is essential for expressing T3SS genes required for bacterial virulence and successful colonization of host plants, our observation that *hrpL* was suppressed by tizoxanide suggests that tizoxanide may effectively inhibit the T3SS-mediated pathogenicity in *E. amylovora*.

Tizoxanide had effects on amylovan production, cell motility, and biofilm formation

Beyond the T3SS inhibition activity of tizoxanide, we further investigated whether tizoxanide has effects on other virulence factors of *E. amylovora*, including amylovan production, cell motility, and biofilm formation. When *E. amylovora* was treated with 6 and 25 µg/mL of tizoxanide, the amylovan production was reduced by 14% and 19% compared to the DMSO control, respectively (Fig. 3a). At the same treatment concentrations, swarming motility was also inhibited by 34% and 67%, respectively, with no effect on bacterial growth (Fig. 3b). Quantification of biofilm formation using crystal violet staining revealed that tizoxanide inhibited biofilm formation by 31% and 48% at concentrations of 6 and 25 µg/mL, respectively,

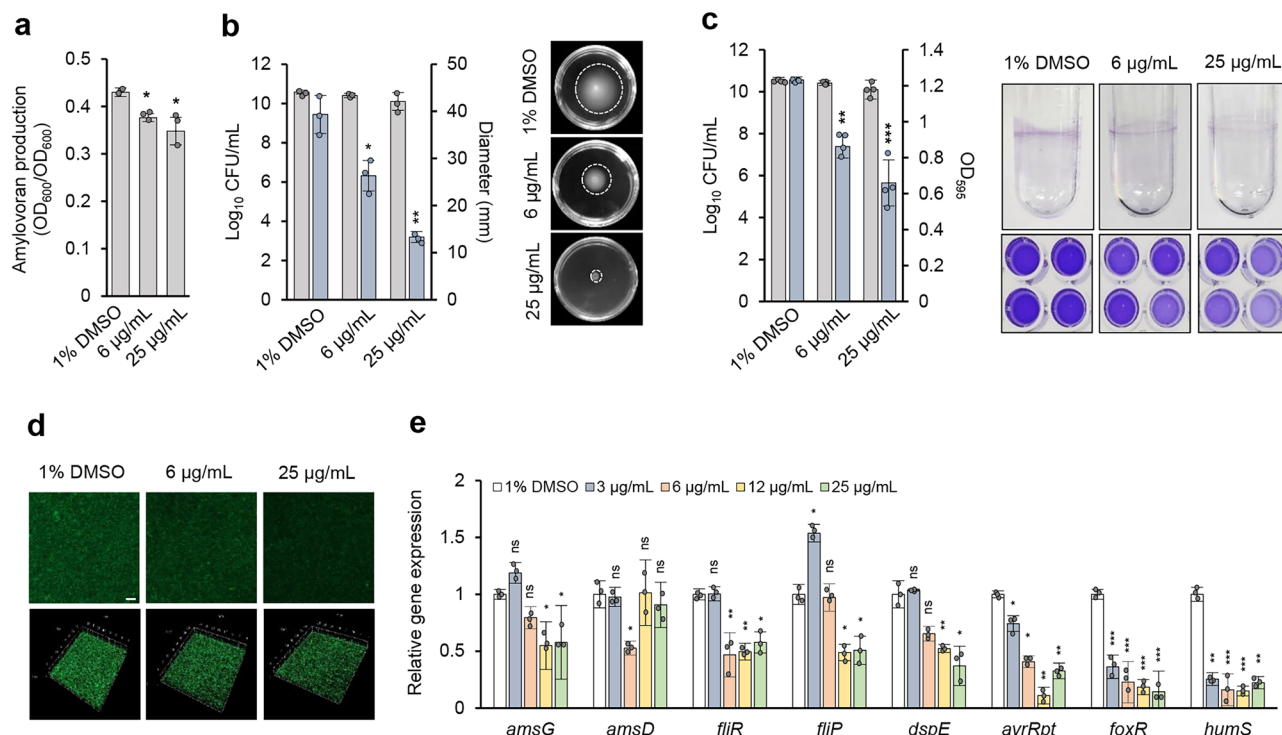


Fig. 3 | Effects of tizoxanide on other virulence factors of *E. amylovora*.

a Amylovoran production of *E. amylovora* TS3128 treated with tizoxanide. Amylovoran production was measured from the culture grown in MBMA medium containing 1% sorbitol and tizoxanide after a 48-h incubation at 28 °C. **b** Swarming motility of *E. amylovora* TS3128 treated with tizoxanide. Bacterial cells were spotted on SMM agar plates containing tizoxanide, and then, the diameter of bacterial spread was measured at 48 h post-inoculation. **c** Crystal violet-based biofilm assay. Biofilm from the cultures grown in LB medium containing tizoxanide for 48 h at 28 °C was quantified and visualized by crystal violet staining assay. **d** Confocal microscope

images of the biofilm accumulation. Biofilm images were captured from *E. amylovora* strain containing pBAV1K-T5-gfp grown in LB medium containing tizoxanide for 6 h at 28 °C. The upper and lower panels were 2D and 3D biofilm accumulation, respectively. Scale bar, 40 µm. **e** Relative expression of genes involved in virulence factors of *E. amylovora* treated with different concentrations of tizoxanide. Total RNA was isolated from the *E. amylovora* TS3128 grown in induction medium for each virulence factor supplemented with tizoxanide. ns, not significantly different; *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$ based on Student's *t*-test.

compared to the DMSO-treated control (Fig. 3c). In addition, we evaluated the ability of *E. amylovora* to accumulate biofilms in the presence of tizoxanide. In the DMSO-treated control, cells formed uniformly robust and multilayered biofilms (Fig. 3d). However, biofilm accumulation in the tizoxanide-treated sample was progressively reduced and appeared less dense (Fig. 3d). This reduction in biofilm accumulation following the tizoxanide treatment suggests that tizoxanide affects the biofilm formation process. Because tizoxanide influences phenotypic traits associated with the virulence factors of *E. amylovora*, we measured the expression of genes related to virulence: amylovoran biosynthesis (*amsG* and *amsD*), motility (*fliR* and *fliP*), T3SS effectors (*dspE* and *avrRpt*), ferrioxamine receptor (*foxR*), and haemin ABC transporter (*hmuS*)^{21,26}. Of the tested genes, the expression of most genes was suppressed at all the tested concentrations of tizoxanide, except for *amsD*. Specifically, the expression of the *foxR* and *hmuS* genes significantly decreased by more than two-folds at a concentration of 3 µg/mL (Fig. 3e). Taken together, our results suggest that tizoxanide not only interfered with T3SS-related genes but also broadly impacted amylovoran production, motility, and biofilm formation, highlighting its potential as a multifaceted anti-virulence agent.

T3SS inhibitors have demonstrated their ability to attenuate virulence factors in various bacterial species. For example, Yang et al. reported that a salicylidene acylhydrazide compound ME0054 suppresses T3SS and amylovoran production in *E. amylovora* compared to a DMSO control²¹. In *Ralstonia solanacearum*, a natural compound umbelliferone suppresses multiple T3SS genes, including *hrpG*, and inhibits biofilm formation, thereby controlling bacterial wilting disease by *R. solanacearum*²⁷. It has been reported that the hydroxyquinoline compound INP1750, a T3SS inhibitor in *P. aeruginosa*, inhibits toxin secretion and flagellar motility and

impairs the activity of the YscN ATPase from *Yersinia pseudotuberculosis*²⁸. These pleiotropic effects of anti-virulence compounds suggest that the inhibitors may target interconnected regulatory pathways involved in bacterial virulence factors and also reduce the risk of resistance development by targeting diverse pathways rather than a specific bacterial function²⁹. Therefore, the discovery of small molecules affecting various virulence factors can lead to disrupting bacterial pathogenicity at multiple levels.

Tizoxanide is involved in controlling the expression of diverse proteins in *E. amylovora*

Considering the broad impact of tizoxanide on critical virulence mechanisms, proteomic analysis of the *E. amylovora* wild-type strain was performed for which the bacterial cells were treated with tizoxanide and incubated for 8 h. A total of 638 and 645 proteins were detected across three biological replicates in the absence and presence of tizoxanide, respectively (Supplementary Data 1). The proteomic analysis revealed that 47 proteins were exclusively detected in the DMSO control, whereas 54 proteins were unique to tizoxanide-treated *E. amylovora* (Fig. 4a). In particular, virulence-related proteins, including type II toxin-antitoxin system RatA family toxin (QKZ10113) and type VI secretion system ATPase TssH (QKZ10416), were specifically detected from the DMSO-treated control (Supplementary Data 2), whereas various proteins associated with regulation and transcription were detected in the tizoxanide-treated *E. amylovora* (Supplementary Data 3).

The quantitative analysis identified 48 proteins highly expressed in the DMSO-treated control and 55 proteins in the tizoxanide-treated *E. amylovora* (Supplementary Data 4, 5). Proteins were more abundant in the presence of tizoxanide belonging to eight categories: C (energy production

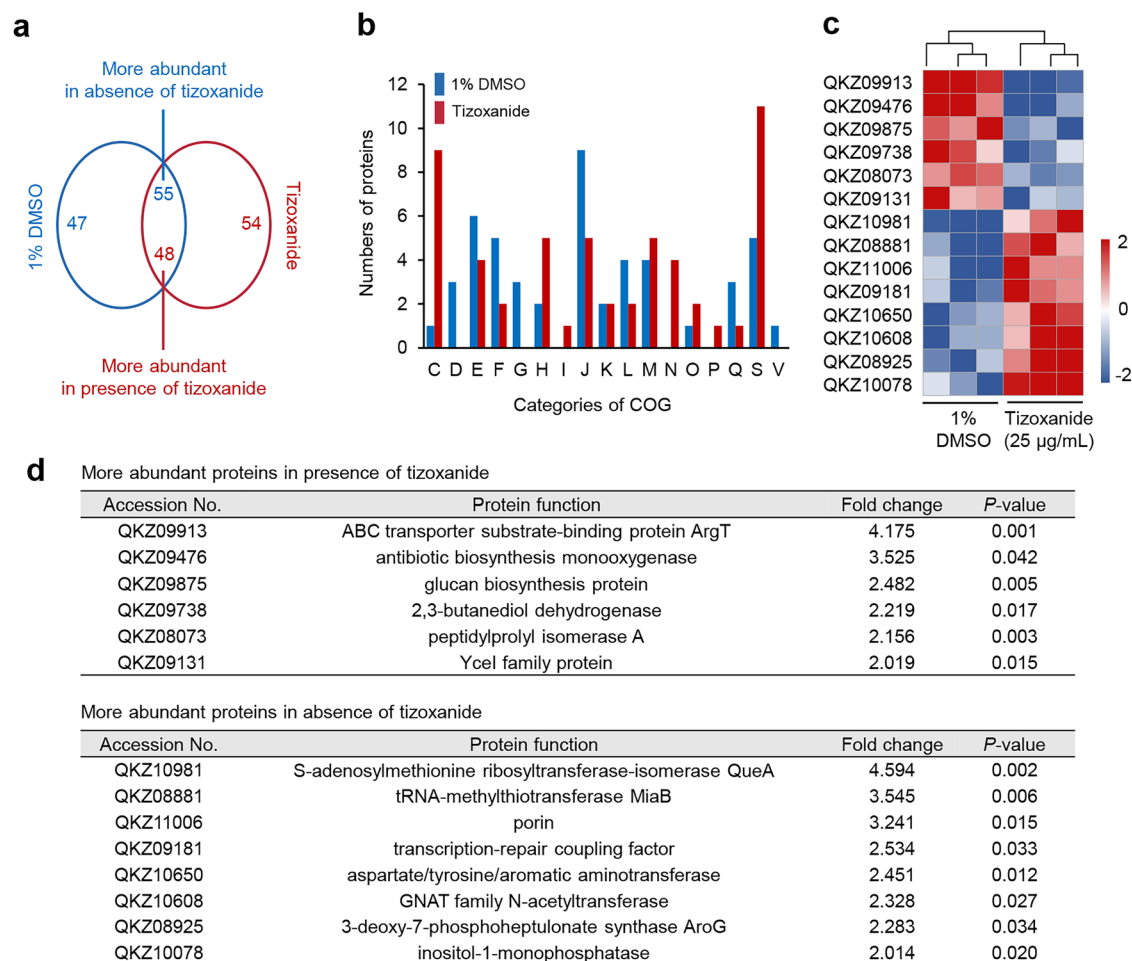


Fig. 4 | Proteomic analysis of *E. amylovora* TS3128 treated with tizoxanide.

a Comparison of differentially expressed proteins in absence or presence of tizoxanide. Bacterial cells were inoculated in LB broth medium with 1% DMSO or 25 µg/mL of tizoxanide. Bacterial cells were harvested at 8 h and then disrupted to obtain the total soluble proteins. The numbers in the Venn diagrams represent the different abundant numbers of proteins (>1.5-fold) based on comparative analyses. **b** Classification of differentially abundant proteins based on the clusters of orthologous groups (COG). Bar graphs indicate the number of classified proteins detected in cultures grown in the absence or presence of tizoxanide. **c** Heatmap of significantly abundant proteins in the

absence or presence of tizoxanide, which was generated by log₂ fold changes. C, energy production and conversion; D, cell cycle control and mitosis; E, amino acid metabolism and transport; F, nucleotide metabolism and transport; G, carbohydrate metabolism and transport; H, coenzyme metabolism; I, lipid metabolism; J, translation; K, transcription; L, replication and repair; M, cell wall/membrane/envelope biogenesis; N, cell motility; O, post-translational modification, protein turnover, and chaperones; P, inorganic ion transport and metabolism; Q, secondary structure; S, function unknown; and V, defense mechanisms. **d** List of proteins with more than two-fold abundance detected in cultures grown with or without tizoxanide.

and conversion), H (coenzyme metabolisms), I (lipid metabolism), M (cell wall/membrane/envelope biogenesis), N (cell motility), O (post-translational modification, protein turnover, chaperones), P (inorganic ion transport and metabolism), and S (function unknown) (Fig. 4b). Notably, group C was more abundant in the tizoxanide-treated *E. amylovora* except for group S. Conversely, proteins were more abundant in the DMSO-treated control belonging to six categories: D (cell cycle control, cell division, chromosome partitioning), E (amino acid transport and metabolism), F (nucleotide transport and metabolism), G (carbohydrate transport and metabolism), J (translation, ribosomal structure and biogenesis), L (replication, recombination, and repair), and Q (secondary metabolites biosynthesis, transport, and catabolism) (Fig. 4b).

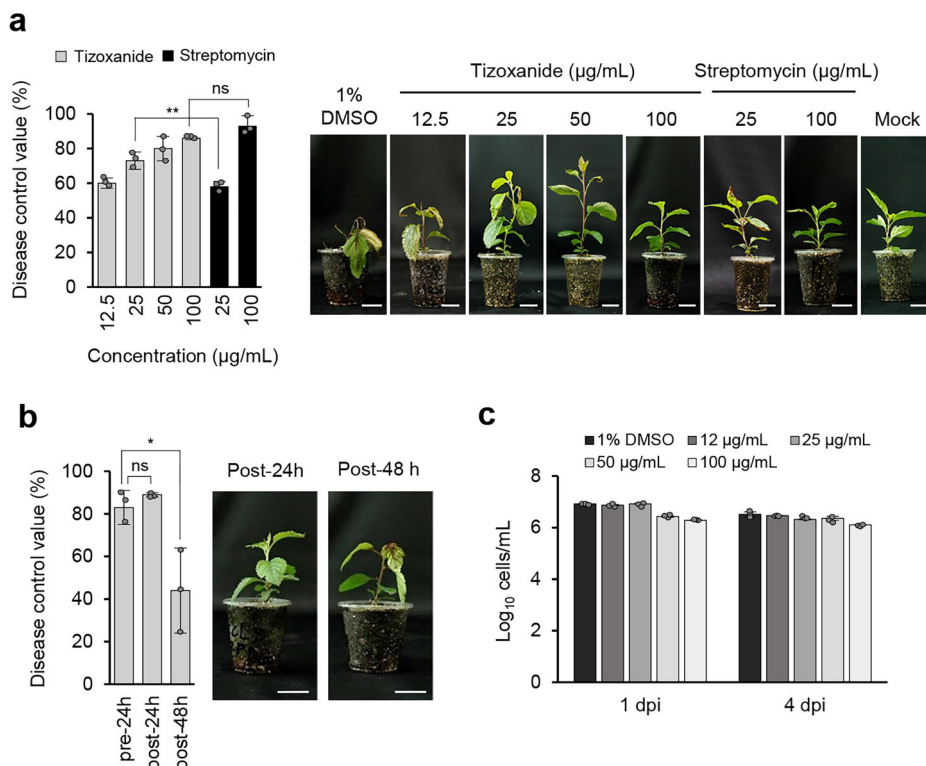
Comparative proteomic analysis identified 14 proteins with differential expression (more than a 2-fold change) between the samples (Fig. 4c). Six proteins were highly expressed in the presence of tizoxanide: lysine/arginine/ornithine ABC transporter substrate-binding protein ArgT (QKZ09913), antibiotic biosynthesis monooxygenase (QKZ09476), glucan biosynthesis protein (QKZ09875), 2,3-butanediol dehydrogenase (QKZ09738), peptidylprolyl isomerase A (QKZ08073), and Ycel family protein (QKZ09131) (Fig. 4d). In contrast, eight proteins were highly

expressed in DMSO-treated control: tRNA preQ1(34) S-adenosylmethionine ribosyltransferase (QKZ10981), tRNA (N6-isopentenyl adenosine(37)-C2)-methylthiotransferase MiaB (QKZ08881), porin (QKZ11006), transcription-repair coupling factor (QKZ09181), aspartate/tyrosine/aromatic aminotransferase (QKZ10650), GNAT family N-acetyltransferase (QKZ10608), 3-deoxy-7-phosphoheptulonate synthase AroG (QKZ08925), and inositol-1-monophosphatase (QKZ10078) (Fig. 4d). Therefore, our proteomic analysis revealed that tizoxanide modulates bacterial cellular proteins involved in energy metabolism, cell wall/membrane biogenesis, and translation in *E. amylovora*.

In terms of virulence factors, we found that OmpR (QKZ08048) was induced by tizoxanide. Considering that OmpR negatively regulates *hrpL* and *hrpN* expression and amylovan production in *E. amylovora*³⁰, OmpR induced by tizoxanide likely suppresses *hrpL* expression, which seems to inhibit T3SS-related genes. In contrast, tRNA-methylthiotransferase MiaB (QKZ08881) was highly expressed in the DMSO-treated *E. amylovora* compared with the tizoxanide-treated *E. amylovora*. MiaB with MiaA forms a two-step enzymatic pathway responsible for one of the most ubiquitous tRNA modifications, which has an important role in stress response and the expression of virulence factors or phenotypes in bacterial pathogens^{31,32}.

Fig. 5 | Disease control efficacy of tizoxanide against fire blight caused by *E. amylovora*.

a Control values of tizoxanide against fire blight on Chinese pearleaf crabapple seedlings. Treatments of streptomycin and 1% DMSO were used as positive and negative controls, respectively. One day after the compound treatment, plants were inoculated with a cell suspension (4×10^8 CFU/mL) of *E. amylovora* TS3128. Disease severity was investigated at 9 dpi, and photos were also taken at 9 dpi. **b** Disease control value of tizoxanide (100 μ g/mL) for different time points of the treatment. pre-24 h, tizoxanide treatment 24 h before inoculation; post-24 h and post-48 h, tizoxanide treatment 24 h and 48 h after inoculation, respectively. **c** Effect of tizoxanide on cell viability onto leaves of Chinese pearleaf crabapple seedling. *E. amylovora* TS3128 (1×10^7 CFU/mL) was spray-inoculated onto the leaf of Chinese pearleaf crabapple seedlings after tizoxanide treatment, and then the randomly selected plant pieces were used for the measurement of viable bacterial cell numbers. ns, not significantly different; *, $P < 0.05$; and **, $P < 0.01$ based on Student's *t*-test. Scale bars, 2 cm.



Previous studies have shown that MiaB positively regulates the expression of T3SS genes in *P. aeruginosa* through the LadS-Gac/Rsm signaling pathway³³. However, the function of MiaB in *E. amylovora* remains unclear. Considering its established role in virulence regulation in other bacterial pathogens, our results suggest that the tizoxanide-induced suppression of MiaB might affect the T3SS master regulator *hrpL* or T3SS-related genes.

The EPS, including amylovan, are essential virulence factors in *E. amylovora*. Amylovan facilitates biofilm development within the plant xylem vessel, leading to vessel blockage and resulting in wilting symptoms³⁴. Given that tizoxanide inhibited both amylovan production and biofilm formation, our proteomic analysis revealed that the phosphotransferase system (PTS) fructose transporter subunit IIA/HPr protein (QKZ09833) and IIBC protein (QKZ09831) were more abundant in the DMSO-treated control. Many carbohydrates are transported into bacterial cells through the PTS for use as energy sources³⁵. Mao et al. demonstrated that PtsH, a key component of PTS, is involved in carbohydrate utilization and also regulates the expression of T3SS genes in *Edwardsiella piscicida*³⁶. This finding suggests that PtsH has a crucial role in coordinating carbohydrate metabolism with virulence gene expression in *E. piscicida*³⁶. In addition, we found that proteins related to lipopolysaccharides, which serve as a physical barrier and mediate biofilm development, were more abundant in the DMSO-treated control. Therefore, our results showed that tizoxanide has broad effects on various virulence factors in *E. amylovora*.

Tizoxanide effectively controlled fire blight on Chinese pearleaf crabapple seedlings

To determine whether tizoxanide could control fire blight disease in vivo, we applied tizoxanide to Chinese pearleaf crabapple seedlings before inoculation of *E. amylovora*. From the DMSO-treated groups, we observed complete necrosis of the terminal buds, petioles, stems, and leaves (Fig. 5a). However, plants treated with the positive control antibiotic streptomycin exhibited a disease control efficacy of 89% at a concentration of 100 μ g/mL compared to the DMSO-treated control (Fig. 5a). When tizoxanide was applied at a concentration of 100 μ g/mL, necrosis was limited on leaf rudiment and leaf blades, achieving a disease control value of 86%. Even at the lowest concentration (12.5 μ g/mL) used in the experiment, tizoxanide

exhibited a disease control efficacy of 60%. Notably, tizoxanide exhibited a disease control value with 73%, which is a better efficacy compared to the positive control, streptomycin, at a concentration of 25 μ g/mL (Fig. 5a).

To investigate whether tizoxanide exhibits curative effects on plants, we inoculated *E. amylovora* on Chinese pearleaf crabapple seedlings before treatment with tizoxanide. When tizoxanide was treated 24 h after pathogen inoculation, the disease control efficacy was 85%, which is similar to that of the control value derived from the treated plants before inoculation (Fig. 5b). However, when tizoxanide was treated 48 h after pathogen inoculation, the treated plants showed complete necrosis on the terminal buds, petioles, and leaves, suggesting that tizoxanide did not exhibit curative effects on plants 48 h after pathogen inoculation (Fig. 5b). The reduced curative effects can be explained by the colonization of the pathogen within plant tissues by 48 h post-inoculation. Tizoxanide is thought to exert its activity, at least in part, by interfering with bacterial secretion systems or early-stage virulence mechanisms, which are most active during the initial phases of host colonization. Once systemic infection or tissue damage has occurred, the compound may be less effective at reversing disease progression. In addition, plant physiological responses, such as defense signaling and cellular damage, may already be underway by 48 hpi, limiting the compound's accessibility or effectiveness at the infection sites. When we explored the microbial population affected by tizoxanide on the plants, there was no significant difference in the number of *E. amylovora* cells on the leaves 1- and 4-days post-inoculation (dpi) (Fig. 5c). Taken together, our results demonstrate that tizoxanide can effectively suppress the development of fire blight and mitigate fire blight symptoms in the Chinese pearleaf crabapple seedling model, without showing bactericidal activity based on microbial cell viability in the presence of tizoxanide.

Improvement of in vitro and in vivo activity of tizoxanide

To enhance the biological activity of tizoxanide, we synthesized derivatives of tizoxanide and evaluated their inhibitory activity of GFP expression controlled by the *hrpA* promoter. To this end, we introduced various substituents on the thiazole and phenol moieties of tizoxanide; consequently, we chemically synthesized 24 derivatives of tizoxanide (Fig. 6 and Supplementary Fig. 4). When an acetyl group was introduced at the phenol

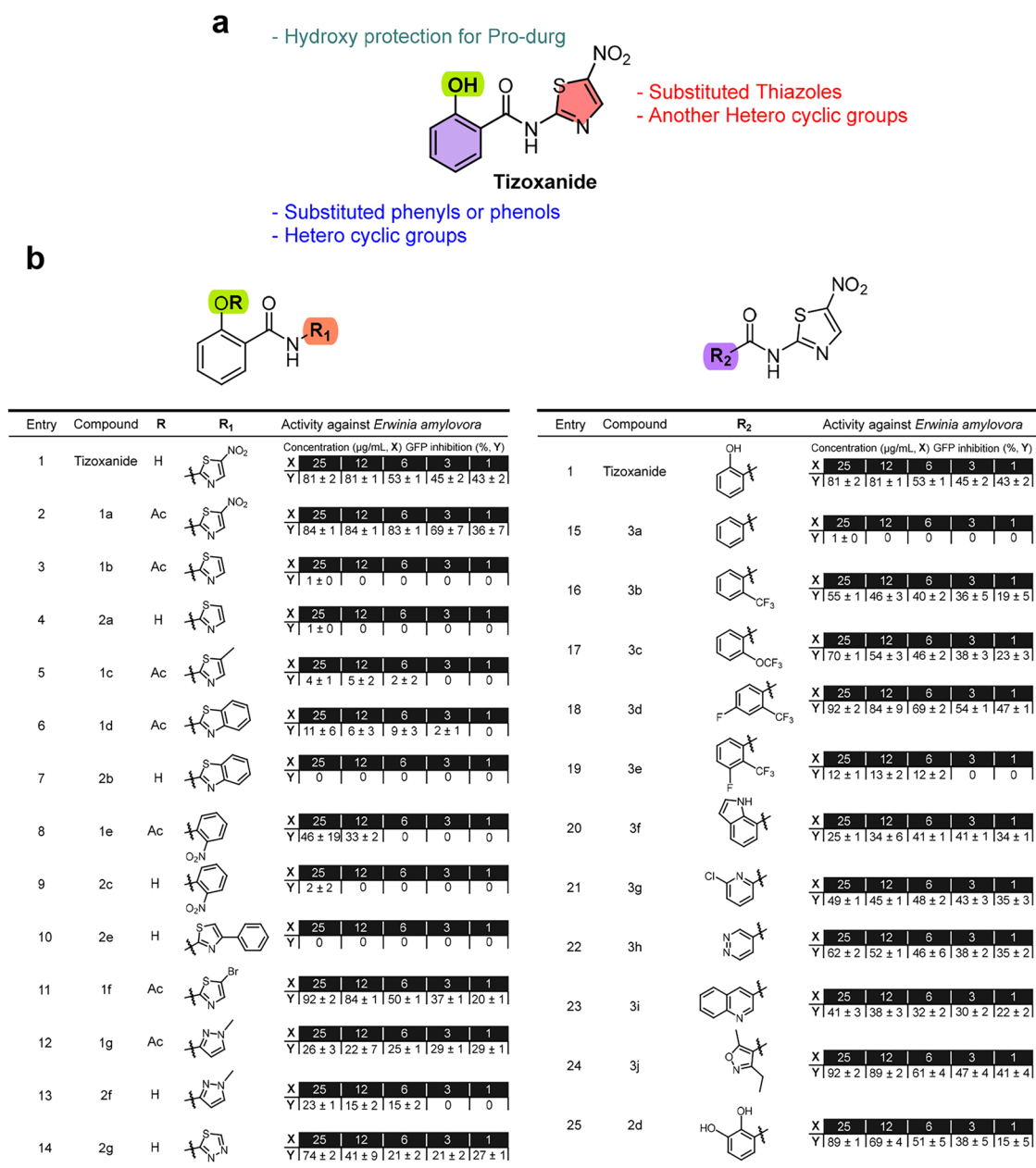


Fig. 6 | Structure-activity relationship of tizoxanide based on the *hrpA* promoter activity. a Synthetic strategy for the generation of 24 tizoxanide derivatives. **b** Inhibitory activity of tizoxanide derivatives for GFP expression controlled by *hrpA*

promoter. GFP inhibition was investigated at 18 h post-inoculation in *E. amylovora* HK0955 grown at 18 °C in HMM medium supplemented with tizoxanide derivatives.

position, the resulting compound nitazoxanide that is known as a pro-drug of tizoxanide (Entry 2)^{37,38} exhibited improved GFP inhibition compared to tizoxanide below a concentration of 6 μg/mL. Compounds lacking the -NO₂ group (Entries 3 and 4) lost their activity, indicating a critical role of the -NO₂ group in tizoxanide. To investigate the impact of hydrophobic interactions, we introduced various substitutions on the thiazole ring, such as methyl (Entry 5), benzothiazole (Entries 6 and 7), and phenyl (Entry 10) substitutions, and the resulting derivatives lost their activity; these results suggest the crucial role of the -NO₂ group on the thiazole ring. However, when we replaced the -NO₂ with a -Br (Entry 11), the compound remained active, suggesting that polar groups at the 5-position of the thiazole are vital for the activity. For further derivatives, we replaced the thiazole scaffold with a nitro-phenyl group (Entries 8 and 9), which resulted in a reduced activity for GFP expression. This finding confirmed the importance of the thiazole scaffold itself in maintaining activity. In addition, changing the thiazole group to pyrazole (Entries 12 and 13) or thiadiazole (Entry 14) did not

improve the activity, further emphasizing the significance of the original thiazole structure.

The phenolic moiety in tizoxanide is known to be inherently unstable due to its susceptibility to various metabolism processes, including oxidation, hydrolysis, and isomerization³⁹. In this study, the complete removal of the phenolic hydroxyl group (Entry 15) resulted in a total loss of activity. However, strategic bioisosteric replacements with -CF₃ (Entry 16) or -OCF₃ (Entry 17) groups retained a weak activity compared to tizoxanide, suggesting that the fluorinated substituents can act as hydrogen bond acceptors while increasing the chemical stability and lipophilicity. The 4-fluoro derivative (Entry 18) exhibited significantly higher potency compared to both the non-fluorinated tizoxanide and the 3-fluoro analog (Entry 19). This positional effect suggests a specific orientation within the binding pocket, where the 4-fluoro substituent might optimize electronic interactions or induce conformational changes that favor target engagement^{40,41}. Despite the well-known utility of indole as a phenol bioisostere⁴², its

incorporation (Entry 20) resulted in reduced activity. Further exploration of heterocyclic replacements, including pyrimidine (Entry 21), pyridazine (Entry 22), and quinoline (Entry 23), failed to yield improvements in activity compared to the parent phenol. Considering that the isoxazole ring system is a well-established pharmacophore in antimicrobial drug design^{43,44}, we hypothesized that an isoxazole moiety in our series could enhance the antimicrobial activity. When we synthesized an isoxazole-containing analog (Entry 24), the isoxazole derivative exhibited an improved GFP inhibitory activity compared to tizoxanide at concentrations of 12 and 25 µg/mL. Furthermore, the disease control value of this derivative was improved by 10%, compared to that of the tizoxanide (Supplementary Fig. 5). Taken together, our results show that the thiazole scaffold is crucial for maintaining the activity in this class of compounds. In addition, for the enhancement of the tizoxanide activity, the metabolically vulnerable phenolic group can be effectively replaced by other heterocyclic compounds, such as isoxazole, or modified through strategic benzene ring substitutions, including the introduction of -CF₃ and -F groups. These findings provide valuable insights and a clear roadmap for the further optimization of this promising class of compounds as potential antimicrobial agents.

Methods

Bacterial strains and culture media

A wild-type strain *E. amylovora* TS3128 was maintained on mannitol glutamate yeast extract medium (MGY), which contained 10 g/L D-mannitol, 2 g/L L-glutamic acid, 0.5 g/L KH₂PO₄, 0.2 g/L NaCl, 0.2 g/L, 0.2 g/L MgSO₄·7H₂O, and 1 g/L yeast extract, at 28 °C⁴⁵. For T3SS induction, *hrp*-inducing minimal medium with galactose (HMM) was used, consisting of 1 g/L [NH₄]₂SO₄, 1, 0.4 g/L MgCl₂·6H₂O, 0.1 g/L NaCl, 8.7 g/L K₂HPO₄, 6.8 g/L KH₂PO₄, 1.8 g/L galactose²¹. Different media were utilized for specific assays: for amylovan production, swarming motility, and biofilm formation, amylovan production medium (MBMA; 3 g/L KH₂PO₄, 7 g/L K₂HPO₄, 1 g/L [NH₄]₂SO₄, 2 mL glycerol, 0.5 g/L citric acid, 0.03 g/L MgSO₄, and 1% sorbitol), swarming motility medium (SMM; 10 g/L tryptone, 10 g/L NaCl, and 3 g/L agar), and Luria Bertani medium (LB; 5 g/L yeast extract, 10 g/L tryptone, and 10 g/L NaCl) were used, respectively^{21,46}. A GFP-expressing *E. amylovora* strain containing pBAV1K-T5-gfp (Addgene, Watertown, MA, USA) provided by Dr. Duck Hwan Park (Gangwon National University, South Korea) was used for the biofilm accumulation assay. When necessary, antibiotics were added to the medium at the following concentrations: 100 µg/mL ampicillin or 100 µg/mL kanamycin.

Generation of the transgenic strains

To find small molecules exhibiting T3SS inhibition activity against *E. amylovora*, we generated a transgenic strain containing a construct with the *hrpA* promoter fused to GFP. To this end, the *hrpA* promoter was amplified from the genome of *E. amylovora* TS3128, incorporating the *EcoRI* and *BamHI* restriction enzyme sites. The amplified *hrpA* promoter was cloned into the promoter-less GFP plasmid pFPV25 (Addgene). The resulting *phrpA* promoter-GFP vector was then transformed into *E. amylovora* TS3128, generating a transgenic strain designated HK0955. The primers used in the generation of the transgenic strains were listed in Supplementary Table 1.

Screening procedures based on *hrpA* promoter activity

The chemical library (2485 compounds) was provided by the Chemical Bank of Korea Research Institute of Chemical Technology (Daejeon, South Korea). Each chemical was provided as 5 µL of an approximately 5 mM solution in DMSO. For the initial screening, the HK0955 strain containing *hrpA* promoter-GFP fusion plasmid was washed with phosphate-buffered saline (PBS) and resuspended in HMM medium. Each chemical (1 µL; 5 mM) and bacterial cell suspensions (199 µL; 3×10^8 CFU/mL) were added into 96-well plates, and 1% DMSO treatment was used as a negative control. GFP intensities were measured with a Synergy LX Multimode Microplate Reader (BioTek, Winooski, Vermont, USA) after 18 h of incubation at 18 °C. For further confirmation, the GFP intensities of the selected

compounds from the initial screening were measured using two-fold serial dilutions, starting at a concentration of 100 µg/mL. To visualize GFP inhibition by the compounds, microscopic observation was performed with a BX53 biological microscope (Olympus, Tokyo, Japan) equipped with a U-HGLGPS GFP filter.

Pyruvate dehydrogenase enzyme assay

E. amylovora TS3128 grown in LB medium for 24 h at 28 °C was harvested and washed twice with PBS. Cells were resuspended in LB medium to an optical density (OD₆₀₀) of 0.2, and tizoxanide was added at final concentrations of 6 and 25 µg/mL. Cultures were incubated for 6 h at 28 °C and harvested by centrifugation. Cell pellets were resuspended in 5 mL of PDH assay buffer and permeabilized with 50 µL of chloroform. The permeabilized cells were then subjected to the pyruvate dehydrogenase activity assay kit (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's instructions. Absorbance was measured at 450 nm using a microplate spectrophotometer (Bio-Rad, Hercules, CA, USA). All assays were performed in triplicate.

In vitro antibacterial activity assay

For the antibacterial activity assay, a bacterial suspension (2×10^5 CFU/mL in MGY medium) of *E. amylovora* TS3128 was added to the wells of a 96-well microtiter plate. Each selected compound was serially diluted two-fold, starting with a concentration of 100 µg/mL in MGY medium. The microplates were incubated at 28 °C for 24 h under static conditions. After incubation, the OD₆₀₀ of each well was measured using a microplate spectrophotometer (Bio-Rad). Controls included wells with 1% DMSO treatment and MGY medium only. The percentage of cell growth was calculated as follows: cell growth (%) = $100 \times [(OD_{600} \text{ of tested compound treatment}) / (OD_{600} \text{ of 1\% DMSO treatment})]$.

RT-qPCR assay

To investigate gene expression after the chemical treatments, total RNA was extracted using the AccuPrep® Bacterial RNA extraction kit according to the manufacturer's instructions (Bioneer, Daejeon, South Korea). Total RNA was quantified using a Nanodrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), and reverse transcription was performed using a cDNA synthesis kit (LeGene Biosciences, San Diego, CA, USA). RT-qPCR analysis was conducted with SYBR® Green Realtime PCR Master Mix (Thermo Fisher Scientific) on a QuantStudio™ Real-Time PCR system (Thermo Fisher Scientific). Three technical repeats were performed for each sample. The 16S rRNA gene was used as a reference gene for normalization, and relative expression levels were calculated through the $2^{-\Delta\Delta CT}$ method⁴⁷. The primers for RT-qPCR assay were listed in Supplementary Table 1.

Measurement of amylovan production

Amylovan production was measured using a modified turbidity assay with cetylpyridinium chloride (CPC) as described previously⁴⁸. Briefly, *E. amylovora* TS3128, grown in LB broth for 24 h, was harvested by centrifugation at 10,000 rpm for 5 min and then washed three times with PBS. The bacterial cells were resuspended in MBMA medium and adjusted to an OD₆₀₀ of 0.2. Tizoxanide was added to the bacterial suspension at final concentrations of 6 µg/mL and 25 µg/mL, with 1% DMSO used as a negative control. The cultures were incubated at 28 °C with shaking for 48 h, after which the supernatant was collected by centrifugation. CPC solution (50 µL; 50 mg/mL) was added to 1 mL of the supernatant, and the mixture was incubated at 28 °C for 10 min. Amylovan concentration was determined by measuring turbidity at OD₆₀₀. The amylovan production levels were normalized based on cell density. Each treatment was performed in triplicate with three independent repeats.

Swarming motility assay

E. amylovora TS3128 was cultured in LB broth for 24 h and washed three times with PBS. The cells were resuspended in PBS to an OD₆₀₀ of 0.5. The cell suspension (5 µL) was spotted at the center of an SMM agar plate

containing tizoxanide and incubated at 28 °C for 48 h. Motility was assessed by measuring the diameter of the bacterial spread on the plate. For the cell viability assay, bacterial cells grown on SMM agar plate were harvested and resuspended in PBS buffer. The cell suspension was then spread onto fresh SMM agar plates supplemented with tizoxanide. After incubation at 28 °C for 24 h, colonies were counted to assess bacterial viability. This assay was performed in triplicate with two repeats.

Biofilm formation assay

To quantify the biofilms of *E. amylovora* TS3128, a crystal violet staining assay was performed as previously described with minor modification⁴⁸. Briefly, each well of a 96-well polystyrene microtiter plate (SPL Life Science, Pocheon, South Korea) was inoculated with 200 µL of culture diluted to an OD₆₀₀ of 0.2 in LB medium containing tizoxanide. After static incubation at 28 °C for 48 h, the suspensions in the wells were carefully discarded, and a 1% crystal violet solution was added to each well to stain the biofilms for 30 min. The stained biofilms were quantified using an elution solution (40% methanol and 10% glacial acetic acid), which was added to resuspend the crystal violet stain. The solubilized crystal violet was measured at an absorbance of 595 nm using a microplate spectrophotometer (Bio-Rad). For visualization, biofilm formation and staining were performed in polystyrene round-bottom test tubes using the same method as described above. This assay was performed in quadruplicate with two repeats.

Biofilm accumulation in flow cells

A GFP-expressing strain *E. amylovora* grown in LB medium was adjusted to an OD₆₀₀ of 0.3, and then, tizoxanide was supplemented at a concentration of 6 and 25 µg/mL. Flow channels in a µ-Slide VI 0.5 glass bottom slide (Ibidi, Gräfelfing, Germany) were pre-conditioned with LB medium for 3 h prior to the assay⁴⁹. Each culture was separately inoculated into flow channels and incubated at 28 °C for 1 h. Subsequently, the cultures were introduced into the flow channels under continuous flow generated by a peristaltic pump (Eyela, NY, USA) for 6 h. Biofilm accumulation in the flow channels was visualized using a Zeiss LSM 800 confocal laser scanning microscope (Carl Zeiss, Baden-Württemberg, Germany) set to laser excitation wavelength of 488 nm and an emission wavelength of 550 nm. Imaging was performed by acquiring Z-stacks of fluorescent bacterial cells in individual flow channels. Three-dimensional representations of the biofilm distributions were generated using ImageJ software⁵⁰.

Proteomic analysis

E. amylovora strain TS3128 was cultured overnight in LB broth medium at 28 °C. The bacterial cell suspension was then seeded in 100 mL of LB broth medium containing 25 µg/mL of tizoxanide and 1% DMSO, respectively. After 8 h of incubation, bacterial cells were harvested and disrupted using the Ultrasonic Processor (Colo-Parmer, Vernon Hills, IL, USA). Protein extraction, peptide preparation, liquid chromatography-tandem mass spectrometry (LC-MS/MS), peptide identification and quantification, and statistical analysis were performed as described previously⁵¹. Briefly, tryptic-digested peptides (1 µg) from three biological replicates were analyzed by LC-MS/MS. The samples were injected into a split-free nano-LC system (Thermo Fisher Scientific) connected to an LTQ Velos Pro instrument (Thermo Fisher Scientific) equipped with Thermo Proteome Discoverer 1.3 software (ver. 1.3.0.399). Full mass spectrometry spectra were acquired with six data-dependent MS/MS scans and analyzed using the SEQUEST algorithm within the Proteome Discoverer software. The *E. amylovora* TS3128 database (NCBI accession no. CP056034 for chromosome) was used for spectra matches, and the target-decoy method was used to increase the confidence level. The analyzed data were imported into Scaffold 4 (Proteome Software) for comparative analysis. Peptide spectra matches (PSMs) for each protein were normalized to total PSMs. The average normalized PSMs across the three biological replicates were calculated for each protein and used to identify differently abundant proteins. To infer potential mechanisms, clusters of orthologous groups (COG) classification was applied. MS proteomics data were deposited in the ProteomeXchange

Consortium via the PRIDE⁵² partner repository with the dataset identifier PXD060613.

In vivo disease control efficacy

To evaluate the disease control efficacy of tizoxanide against fire blight disease, Chinese pearleaf crabapple seeds (*Malus asiatica* Nakai) were sown and grown in a greenhouse at 25 ± 5 °C for 4–5 weeks. One day before pathogen inoculation, tizoxanide dissolved in DMSO was applied to the plants by spraying. For the post-inoculation assay, tizoxanide (100 µg/mL) was applied by spraying one or two days after pathogen inoculation. All the treatment solutions included 0.025% Tween 20 (w/v) as a wetting agent to ensure uniform application. Streptomycin (100 µg/mL) and 1% DMSO were used as positive and negative controls, respectively. The treated plants were inoculated with a bacterial suspension (3×10^8 CFU/mL) of *E. amylovora* TS3128 grown in an MGY medium. Following inoculation, the plants were incubated in a humidified chamber at 25 °C for two days and then transferred to a growth chamber (25 °C, 80% relative humidity, and 12-h photoperiod). After seven additional days of incubation, disease severity was assessed by modified methods⁴⁵. The disease severity index (DSI) ranged from 0 to 10: 0, no symptom; 1, partial necrosis of the shoot tip; 2, complete necrosis of the shoot tip; 5, complete necrosis on the petiole of terminal leaves; and 10, complete necrosis on the main stem. The disease control value was calculated as follows: control efficacy (%) = $100 \times [1 - (\text{DSI of each treatment} / \text{DSI of DMSO control})]$. All experiments were conducted twice with triplicates.

Assessment of viable *E. amylovora* on plant leaves

To evaluate the microbial population affected by tizoxanide on the plant, the number of *E. amylovora* cells on the leaves of *M. asiatica* was assessed by cell counting. Briefly, plant leaves were treated with tizoxanide at concentrations of 12, 25, 50, and 100 µg/mL. Three hours after tizoxanide treatment, the leaves were inoculated with a bacterial suspension (1×10^7 CFU/mL) of *E. amylovora* TS3128. The inoculated plants were incubated in a humidified chamber at 25 °C. At 1 and 4 dpi, the leaves were aseptically cut into small pieces using sterile scissors, and the bacterial population was quantified using the QUANTOM Tx™ Microbial Cell Counter (Logos biosystems, Anyang, South Korea) according to the manufacturer's protocol. Each measurement was performed in triplicate, with two independent repeats.

Chemical synthesis

All reagents were purchased from commercial suppliers and used without further purification. Unless otherwise noted, reactions were conducted under an argon atmosphere in dry, anhydrous solvents. Analytical thin-layer chromatography was performed on EMD silica gel 60-F plates (0.2 mm), and compound were visualized under UV light at 254 and 365 nm. Flash column chromatography was performed on the Combi Flash® R_f⁺ system equipped with Redi Sep® R_f silica columns (230–400 mesh). Structure characterization was performed using nuclear magnetic resonance (NMR) spectroscopy on Bruker Ultra shield™ 300 MHz, Bruker Ascend™ 400 MHz, or Bruker Ultra shield™ 500 MHz. ¹H NMR spectra were recorded with chemical shifts quoted in parts per million (ppm) referenced to solvent peaks: CDCl₃ (δH = 7.26 ppm), DMSO-*d*₆ (δH = 2.50 ppm), acetone-*d*₆ (δH = 2.05 ppm), MeOD (δH = 3.31 ppm), or tetramethylsilane (δH = 0 ppm). NMR spectra were analyzed using MestReNova 15.0.1, and high-resolution mass spectra (HRMS) were obtained using a JEOL JMS-700 spectrometer in positive ion mode using fast atom bombardment and electron impact. Tizoxanide derivatives (Supplementary Fig. 4) were synthesized according to the general procedures (Supplementary Figs. 6–9; Supplementary Methods) described in Supplementary Information. ¹H NMR spectra and HRMS data for all compounds are provided in Supplementary Figs. 10–34.

Statistics and reproducibility

Quantitative data in this study were obtained from at least two independent experiments, each conducted with three biological replicates unless

otherwise indicated. Data were presented as the mean $\pm$ standard deviation. Statistical analyses were performed using R software (version 4.1.2). A two-tailed unpaired Student's *t*-test was used to compare two groups, with significant differences indicated by asterisks. For the analysis of *hrpA* expression in response to the eight hit compounds, one-way analysis of variance (ANOVA) followed by Tukey's honestly significant difference (HSD) test was performed ($P < 0.05$). Significant differences among groups were denoted by different lowercase letters above each bar.

Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability

The original contributions presented in the study are included in the article/Supplementary material. MS proteomics data were deposited in the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD060613. Unedited gel image can be found in Supplementary Fig. 1. Source data underlying figures in the main text are available in Supplementary Data 6.

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Author contributions

Y.K., J.-H.L., S.-W.H., G.J.C., and H.K. conceived and designed the experiments. Y.K., E.-J.K., D.K., B.K., and J.W.H. performed the experiments. Y.K., J.W.H., J.-H.L., and H.K. processed, analyzed, and visualized the data. Y.K., J.-H.L., S.-W.H., and H.K. wrote the manuscript. All authors read, corrected, and approved the final manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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