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A novel toxoflavin-quenching regulation in bacteria and its application to resistance cultivars

Okhee Choi,[1] Yeyeong Lee,[2] Jiyeong Park,[2] Byeongsam Kang,[3] Hyun Jin Chun,[1] Min Chul Kim[1,3] and Jinwoo Kim[1,2,3]

[1]*Institute of Agriculture and Life Science, Gyeongsang National University, Jinju, 52828, South Korea.*
[2]*Department of Plant Medicine, Gyeongsang National University, Jinju, South Korea.*
[3]*Division of Applied Life Science, Gyeongsang National University, Jinju, South Korea.*

Summary

The toxoflavin (Txn), broad host range phytotoxin produced by a variety of bacteria, including *Burkholderia glumae*, is a key pathogenicity factor of *B. glumae* in rice and field crops. Two bacteria exhibiting Txn-degrading activity were isolated from healthy rice seeds and identified as *Sphingomonas adhaesiva* and *Agrobacterium* sp. respectively. The genes *stdR* and *stdA*, encoding proteins responsible for Txn degradation of both bacterial isolates, were identical, indicating that horizontal gene transfer occurred between microbial communities in the same ecosystem. We identified a novel Txn-quenching regulation of bacteria, demonstrating that the LysR-type transcriptional regulator (LTTR) StdR induces the expression of the *stdA*, which encodes a Txn-degrading enzyme, in the presence of Txn as a coinducer. Here we show that the bacterial $StdR^{Txn}$-quenching regulatory system mimics the $ToxR^{Txn}$-mediated biosynthetic regulation of *B. glumae*. Substrate specificity investigations revealed that Txn is the only coinducer of StdR and that StdA has a high degree of specificity for Txn. Rice plants expressing StdA showed Txn resistance. Collectively, bacteria mimic the mechanism of Txn biosynthesis regulation, employ it in the development of a Txn-quenching regulatory system and share it with neighbouring bacteria for survival in rice environments full of Txn.

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For correspondence. E-mail jinwoo@gnu.ac.kr; Tel. +82 55 772 1927; Fax +82 55 772-1929.
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Introduction

Toxoflavin (Txn) is a bright yellow pigment {1,6-dimethylpyrimido[5,4-*e*]-1, 2,4-triazine-5,7(1*H*,6*H*) dione; molecular weight= 193}, which exhibits antibacterial, antifungal and herbicidal activities and is toxic to animals (Latuasan and Berends, 1961; Sato *et al*., 1989; Iiyama *et al*.,1995; Cox *et al*., 2000; Nagamatsu, 2001; Kim *et al*., 2004). Several bacterial strains are known as Txn producers, including *Burkholderia cocovenenans*, *B. gladioli*, *B. glumae*, *B. plantarii*, *Pseudomonas protegens* and *Streptomyces* spp. (Levenberg and Linton, 1966; Lynch and Dennis, 2010; Machlowitz *et al*., 1954; Philmus *et al*., 2015). It has been reported that *B. cocovenenans* intoxication caused by two toxins, bongkrekic acid and Txn, produced an average of 288 poisonings and 34 deaths in Indonesia between 1951 and 1975 (Latuasan and Berends, 1961; Cox *et al*., 2000). The mode of action of Txn toxicity has been proposed: briefly, under aerobic and visible light conditions, Txn functions as an active electron carrier that is reduced and transfers its electrons to oxygen, resulting in the production of hydrogen peroxide (Latuasan and Berends, 1961).

Burkholderia glumae was isolated from an infant with chronic granulomatous disease (Devescovi *et al*., 2007). *B. glumae* produces the essential key pathogenic toxin, Txn, which is responsible for the development of the major symptoms of panicle blight (grain rot in Japan and Korea) of rice and wilt of many field crops (Jeong *et al*., 2003; Kim *et al*., 2004). The Txn biosynthesis pathway of *B. glumae* has been revealed: the TofRI quorum sensing system and ToxR LysR-type transcriptional regulator (LTTR) in the presence of its coinducer Txn ($ToxR^{Txn}$) positively regulate the expression of both *toxABCDE* (biosynthesis) and *toxFGHI* (exporter) operons (Kim *et al*., 2004; Kim *et al*., 2009).

To attenuate the virulence of phytopathogenic bacteria, quorum-quenching has been suggested as an antivirulence strategy. Lactonase produced by *Bacillus* spp. hydrolysed the lactone bond in the homoserine ring of N-acyl homoserine lactones (AHLs), thereby inactivating the signal molecules, and transgenic plants expressing AHL lactonase have been shown to exhibit resistance to quorum-dependent bacteria (Dong *et al*., 2001). The acylase is another enzyme involved in the quorum-quenching (Bokhove *et al*., 2010). Toxin-quenching is another antivirulence strategy in toxin-mediated diseases. To attenuate

the virulence of *B. glumae*, the *tflA* gene encoding the enzyme Txn lyase was isolated from *Paenibacillus polymyxa* and transferred into *Arabidopsis* and rice plants, resulting in Txn resistance (Koh *et al*., 2011).

Although there have been several studies on the significance of Txn with respect of its pathogenicity and biochemical characteristics, reports on the biodegradation of Txn or the usage of chemical agents including Txn itself for herbicides, pH indicators and anticancer agents are rare. Genes responsible for Txn degradation previously reported include *tflA* of *P. polymyxa* JH2 (encoding 221 aa; amino acid residues; Koh *et al*., 2011), *toxM* of *P. protegens* Pf-5 (137 aa; GenBank accession no. AAY90317; Philmus *et al*., 2015) and metagenome-derived *txeA* (140 aa; GenBank accession no. KT210132; Choi *et al*., 2018). In the present study, two bacteria exhibiting Txn-degrading activity were isolated from healthy rice seeds. Here we show that bacteria mimic the mechanism of Txn biosynthesis regulation, employ it in the development of a Txn-quenching regulatory system and share it with neighbouring bacteria in rice environments full of Txn.

Results

Isolation and identification of the Txn-degrading enzyme

To isolate Txn-degrading bacteria, healthy rice seeds were germinated in AB minimal medium with 40 μg m^{-1} Txn. Two bacterial isolates that survived in the presence of Txn were isolated. The isolated bacteria were identified as *Sphingomonas adhaesiva* SG14 and *Agrobacterium* sp. AL14 based on 16S rRNA gene sequencing (> 99% identities) and whole-cell matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS; score 2.2, species identification; and 1.6, genus identification respectively). To identify the genes responsible for Txn degradation, random mariner-based transposon mutagenesis was performed in *S. adhaesiva* SG14. Seven mutants of *S. adhaesiva* SG14, which exhibited growth deficiency in Txn, were isolated, and their transposon-flanking regions were analysed using touchdown PCR and sequencing. Sequencing revealed that the mutation sites were physically close. Two possible open reading frames, called *stdA* (*Sphingomonas* toxoflavin-degrading gene) and *stdR*, were identified (GenBank accession no. MH253465) in 2.1 kb-DNA regions, which were confirmed by PCR analysis using primers StdB and StdH, and sequencing (Fig. 1). One mutant of *Agrobacterium* sp. AL14, which exhibited growth deficiency in Txn, was isolated, and its transposon-flanking region was analysed. Surprisingly, a gene 100% identical to that of *S. adhaesiva* SG14, annotated as glyoxalase/bleomycin resistance/dioxygenase, was identified (Fig. 1).

The gene *stdA* encodes a protein that expected to be 16.37 kDa with 153 amino acid residues, which was much smaller than the previously reported *tflA* gene encoding 221 amino acid residues (Koh *et al*., 2011). BLASTP analysis showed 97% identity with the vicinal oxygen chelate (VOC) superfamily of *Klebsiella pneumoniae* (GenBank accession no. WP_023288345). VOC is found in a variety of structurally related metalloproteins, including extradiol dioxygenases, glyoxalase and antibiotic resistance proteins. Amino acid sequence analysis of StdA demonstrated 25.81% identity with human glyoxalase I (SWIS-MODEL; template 3vw9.1) (Fig. S1).

The gene *stdR* is 927 bp and encodes a protein expected to be 34 kDa. BLASTP analysis showed 75% identity with the LysR-type transcriptional regulator (LTTR) of *Sphingomonas panacis* (GenBank accession no. WP_069206523). The presence of the putative T-N_{11}-A sequences of the LTTR-binding boxes suggests the regulation of *stdA* expression is under StdR control in *S. adhaesiva* SG14 and *Agrobacterium* sp. AL14 (Fig. 1).

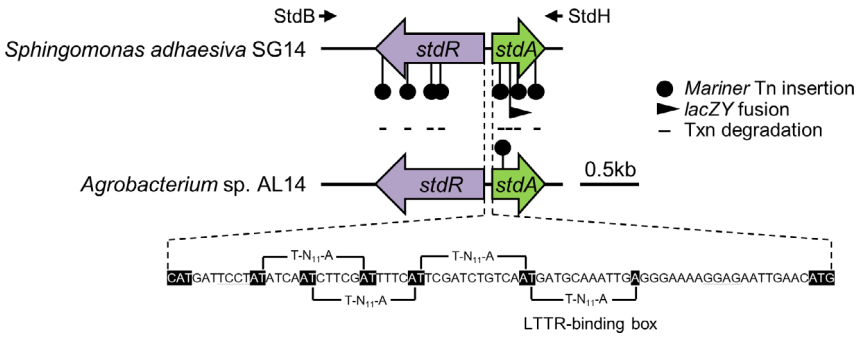

Fig. 1. Organization of *stdRA* genes responsible for Txn degradation. Arrows indicate the positions and orientations of genes responsible for Txn-quenching. Vertical bars with closed circles indicate the position of the *Mariner* Tn insertions, and the major phenotypes of the mutants are represented on the map. A vertical bar with an arrow in the map indicates the position and orientation of the *lacZY* insertion. Highlighted letters represent the T-N_{11}-A sequences of the putative LysR-type transcriptional regulator (LTTR)-binding boxes. The ribosomal binding site is underlined. Using primers StdH and StdB, the region of 2.1 kb-DNA containing *stdR* and *stdA* genes was confirmed by PCR.

stdR *and* stdA *are responsible for Txn degradation*

To further confirm the function of *stdA* and *stdR* in Txn-degrading activity, *lacZY* transcriptional integrations of *stdA* and non-polar deletion mutations of *stdR* were generated in both SG14 and AL14 strains. Wild-type strains were resistant to Txn, whereas *stdA*$^-$ or Δ*stdR* mutants were sensitive. Genetic complementation of *stdA*$^-$ or Δ*stdR* mutants with pCOK399 (pBBR1-MCS5::P_{lac}–*stdA*) or pCOK403 (pSRKGm::P_{lac}–*stdR*) restored Txn resistance respectively (Fig. 2A and C; upper panel). *stdA*$^-$ or Δ*stdR* mutants failed to degrade Txn in AB broth, resulting no growth. Growth deficiency of *stdA*$^-$ or Δ*stdR* mutants in the presence of Txn was recovered by genetic complementation with P_{lac}–*stdA* or P_{lac}–*stdR* respectively (Fig. 2A and C; lower panel). Pale yellow-coloured AB broth indicates no growth of mutant strains or no degradation of toxoflavin. The Txn degradation was confirmed using thin-layer chromatography (TLC) analysis, which was proven to be a simple and effective method for the detection of Txn and its derivatives (Fig. 2B and D).

Biochemical characteristics of the Txn-degrading enzyme, StdA

To determine the biochemical characteristics of the Txn-degrading enzyme StdA, StdA was overexpressed and purified as a His-tagged recombinant protein from *E. coli* BL21(DE3) carrying pCOK393(pET21b::*stdA*). StdA-His was 17.19 kDa (Fig. S2A). The optimum pH and temperature for Txn degradation of StdA-His were pH 8.0–8.5 and 28°C respectively (Fig. 3A and B). StdA-His was stable at high temperature. The Txn-degrading activity of StdA-His was retained from 15 to 55°C (Fig. S2B). The specific activity and K_M values of StdA-His were 0.329 μmol min^{-1} mg^{-1} and 64.98 μM respectively (Fig. 3C). Unlike other metal-dependent extradiol dioxygenases, StdA-His degraded Txn in the absence of metal ions and required DTT for Txn degradation (Fig. 3D).

To investigate the substrate specificity of StdA-His, various Txn derivatives were tested as substrates (Fig. S3). Txn (100%) and reumycin (> 80%) were degraded by 3 μM StdA–His in 10 min at 28°C; however, other derivatives showed no detectable degradation (Fig. 3E). These results indicated that StdA has a high degree of specificity for Txn without a metal ion requirement.

StdR regulates the expression of stdA *in the presence of Txn*

To determine if StdR regulates *stdA* expression, we analysed the expression of *stdA*::*lacZY* in wild-type SG14, Δ*stdR* and *stdR*-complemented strain backgrounds (Fig. 4A). When β-galactosidase activity was measured in each strain, *stdA* expression was abolished

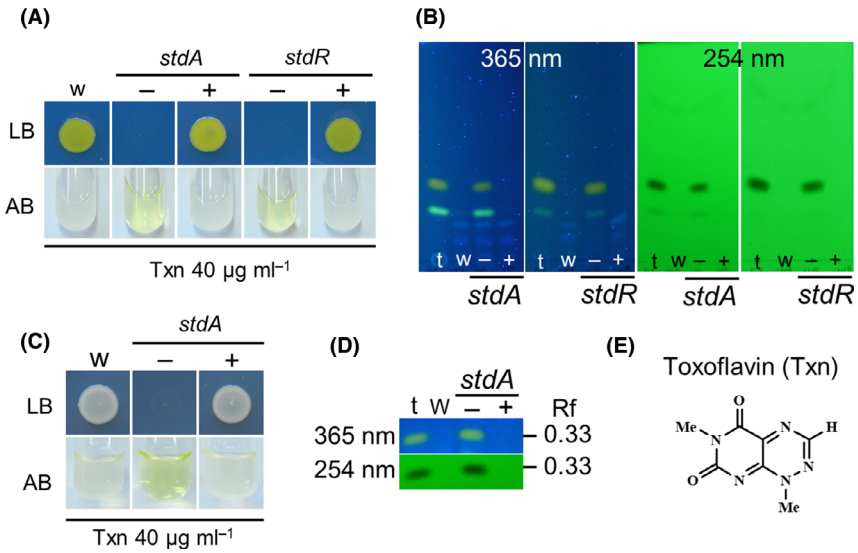

Fig. 2. Effects of wild-type *Sphingomonas adhaesiva* SG14, *Agrobacterium* sp. AL14, and their mutants on Txn-degrading activity.
A. Txn resistance of *S. adhaesiva* SG14 strains on LB agar (upper panel) and in AB liquid culture (lower panel).
B. Thin-layer chromatography analyses of Txn.
C. Txn resistance of *Agrobacterium* sp. AL14 on LB agar (upper panel) and in AB liquid culture (lower panel).
D. TLC analysis of Txn. Rf indicates retardation factor on TLC developed in chloroform:methanol (95:5, v:v). Corresponding spots were visualized under UV wavelength at 254 and 365 nm.
E. Chemical structure of Txn. t, Txn; w, wild type; –, *stdA*$^-$ or Δ*stdR* mutants; and +, genetic complementation of *stdA*$^-$ or Δ*stdR* mutants with pCOK399 (pBBR1-MCS5::P_{lac}–*stdA*) or pCOK403 (pSRKGm::P_{lac}–*stdR*) respectively.

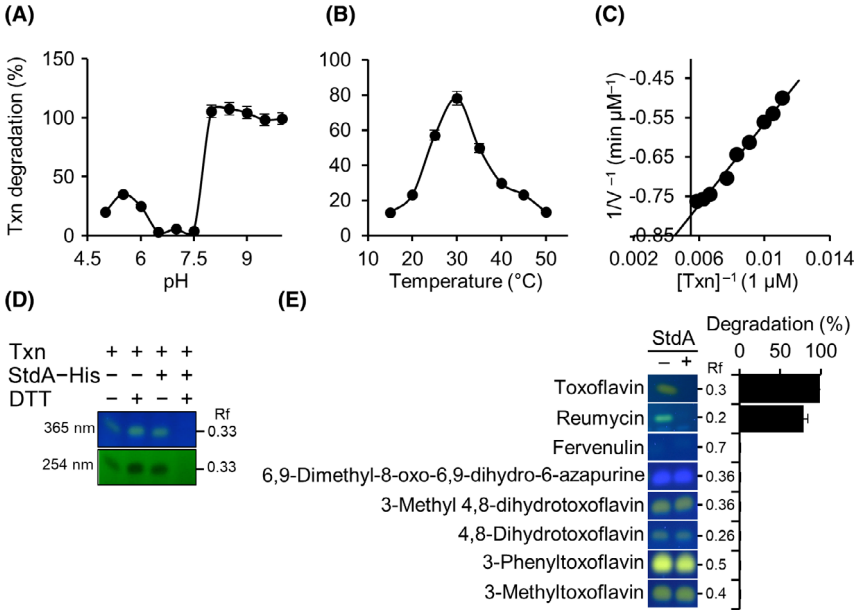

Fig. 3. Characterization of StdA Txn degradation.
A. The optimum pH, 8.0; (B) temperature, 28°C; (C), Lineweaver-Burk plot for Txn degradation by StdA-His. All enzyme assays for temperature were performed in 50 mM sodium phosphate buffer (pH 8.0) as described in the Experimental procedures section. All values are the means ± SD of values from triplicate experiments ($n = 3$).
D. DTT-dependent and metal ion-independent Txn degradation of StdA. *In vitro* Txn degradation assay was performed in 50 mM sodium phosphate buffer (pH 8.0) with 100 μM Txn plus/minus purified StdA–His or plus/minus 5 mM DTT.
E. Substrate specificity of purified StdA–His to Txn and its derivatives. Rf indicates retardation factor on TLC developed in chloroform:methanol (95:5, v:v). Corresponding spots were visualized under UV wavelength at 365 nm.

in the absence of StdR or Txn. StdR induced *stdA* expression in the presence of 1–10 μM Txn (Fig. 4B). Mutant strains were sensitive to Txn at concentrations higher than 10 μM, so concentrations below 10 μM were used. These results indicated that the StdR positively regulates the expression of *stdA*, and StdR requires Txn as a coinducer to activate the expression of *stdA* (Fig. 4B). By combining genetics with gene expression analysis, we propose a novel working model of how bacteria quench Txn (Fig. 4C), demonstrating that bacteria mimic the mechanism of Txn biosynthesis regulation (Fig. 4D) and employ it in the development of the Txn-quenching regulation system.

Characterization of the StdR, Txn-quenching regulator

The specific DNA sequence of *stdR* revealed that StdR exhibits functional domains including DNA binding, Txn recognition/response and oligomerization (Schell, 1993; Kim *et al.*, 2009). The sequence of StdR is similar to that of ToxR of *B. glumae* and contains a helix-turn-helix (HTH) motif in its N-terminal domain (Fig. 5). To determine the regions of StdR important for *stdA* expression, StdR mutants were constructed in a similar manner as the StdA mutants. The StdR mutants were introduced into the Δ*stdR stdA*::*lacZY* mutant strain by conjugation, and their activation activities were analysed via *stdA* expression using β-galactosidase assay. We examined six StdR mutants (P9L, V27A, N35S, P39S, L64P and R69Q) associated with DNA binding, three mutants (L106P, L115R and L191H,) associated with the coinducer recognition/response, and three mutants (W234R, G243E and D288G) associated with the oligomerization-impaired expression of *stdA* (Fig. 5A and B). The results were consistent with the putative Txn binding pockets and oligomerization interfaces predicted based on the sequence-structure comparison with *Burkholderia* sp. DntR structure (1UTH_A) (Kim *et al.*, 2009).

Next, we investigated the coinducer specificity of StdR with Txn and its derivatives (Fig. S3). The expression of *stdA* was induced only in the presence of Txn, indicating that Txn is a specific coinducer of StdR (Fig. 5C).

The stdAR *genes are present in a mobile plasmid*

To determine whether toxoflavin-degrading genes, *stdAR*, are present in the mobile plasmid, we performed conjugation assays using the *stdA*::*lacZY* mutant strain (kanamycin-resistant) as a donor. *Agrobacterium tumefaciencs* C58 harbouring pSRKGm (gentamycin-resistant) was used as a recipient. The conjugation rates of SG14 *stdA*::*lacZY* strain and AL14 *stdA*::*lacZY* strain were

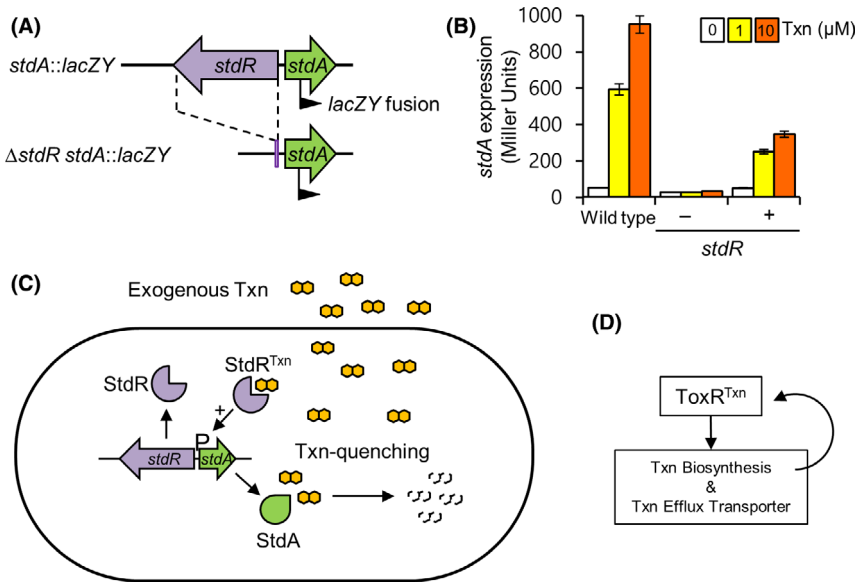

Fig. 4. The StdR/Txn-quenching regulatory system mimics the ToxR/Txn-mediated biosynthetic regulation of *B. glumae*.
A. A *lacZY* insertion in the coding sequence of *stdA* was generated on the genetic background of the non-polar *stdR* deletion mutant strain SG14-OK2 (Δ*stdR stdA*::*lacZY*). The directions of the transcription of *stdR* and *stdA* are indicated by arrows.
B. Expression of *stdA*::*lacZY* in the wild type, Δ*stdR* mutant (–), and complementation strain (+; pCOK403; pSRKGm::P_{lac}–*stdR*) backgrounds supplemented with 500 μM IPTG with or without Txn. The *stdA* was only expressed in the presence of StdR and Txn. Cultures were grown at 28°C in LB medium in the presence or absence of Txn (1 and 10 μM). β-Galactosidase activity was measured as described in the Experimental procedures section. Values are means ± standard deviation (SD) of three replicates.
C. Schematic of the novel Txn-quenching gene regulation identified in two Txn-resistant bacteria, *Sphingomonas adhesiva* and *Agrobacterium* sp., isolated from rice seeds. Exogenous Txn (double hexagon shape) binds to StdR, resulting in $StdR^{Txn}$ complex-dependent activation of the expression of *stdA*, resulting in Txn-quenching. The oval represents a bacterial cell.
D. $ToxR^{Txn}$-mediated autoregulation of Txn biosynthesis and efflux transporter system of *Burkholderia glumae*.

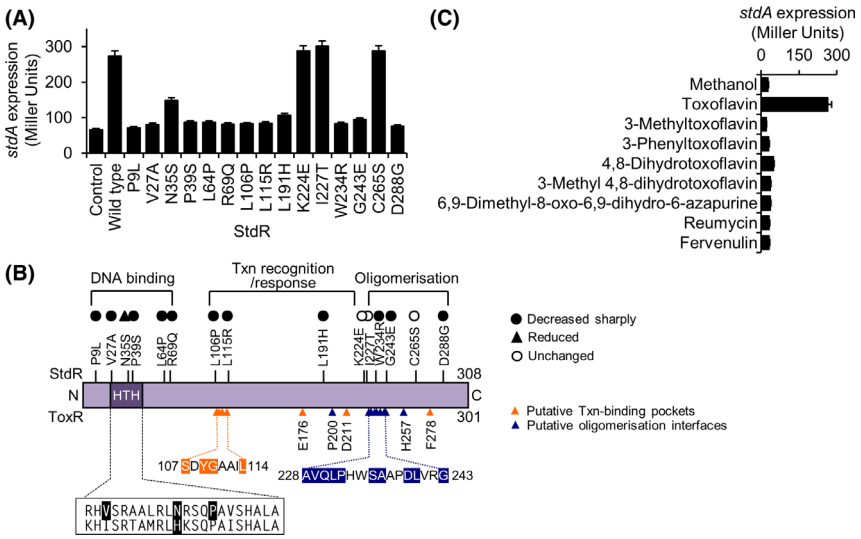

Fig. 5. Characterization of the StdR Txn-quenching regulator.
A. Expression of *stdA*::*lacZY* was regulated by StdR or a single amino acid substituted StdR in the Δ*stdR* mutant background.
B. Distribution of the investigated StdR mutants. The positions of the helix-turn-helix (HTH) motif, Txn recognition/response, and oligomerization proposed to be involved in the inducer response of LTTR are shown in the upper panel. Proposed putative Txn binding pockets and oligomerization interfaces based on the sequence-structure comparison with *Burkholderia* sp. DntR structure (1UTH_A) are shown in the lower panel (Kim *et al.*, 2009; Marchler-Bauer *et al.*, 2017). HTH mutants of StdR and ToxR are highlighted.
C. Coinducer specificity of StdR with Txn and its derivatives. Expression of *stdA*::*lacZY* was regulated by StdR and the coinducer Txn or its derivatives, indicating that Txn is a specific coinducer of StdR. β-Galactosidase activity was repeated three times with duplicate cultures each time (n = 3). The column denotes the data mean, and error bars indicate the range.

2.2×10^{-7} transconjugants per donor and 2.2×10^{-9} transconjugants per donor respectively. Transconjugants showing kanamycin and gentamycin resistance were confirmed using multiplex PCR amplification for simultaneous detection of *stdA* and *virD* genes (Fig. S4). These results suggest that the toxoflavin-degrading genes are present in the plasmid and horizontal transfer occurs.

Txn-quenching is an ideal antivirulence strategy in Txn-mediated diseases

To determine whether transgenic plants expressing *stdA* are resistant to Txn, the pBS43 was constructed for plant transformation. The coding region of *stdA* was amplified and cloned into pCAMBIA 1300PT, which contains the hygromycin resistance gene under the control of the 35S promoter, resulting in pBS43 (Fig. 6A). *Agrobacterium*-mediated rice transformation was performed using rice-calli inoculation with *A. tumefaciens* LBA4404 carrying pBS43. Leaf discs of the T_1 rice transgenic line, Nd43-1, showed resistance against Txn up to 20 μg ml^{-1} (Fig. 6B). Wild-type Nakdongbyeo was sensitive to 5 μg ml^{-1} Txn and showed complete chlorosis in 20 μg ml^{-1} Txn under light conditions. However, the Nd43-1 line showed chlorosis only at the merged regions of leaf discs in 20 μg ml^{-1} Txn (Fig. 6B). These results were confirmed by the quantification of chlorophyll. As the Txn concentration increased, the chlorophyll levels in wild-type rice leaf discs gradually decreased (Fig. 6B). The Nd43-1 line exhibited approximate 60% Txn degradation compared to the wild type (Fig. 6C). Putative T_1 rice transformants were verified using reverse transcription (RT)-PCR with a *stdA* gene primer (Fig. 6D). These results indicated that rice expressing *stdA* may be useful in the development of cultivars resistant to rice grain rot.

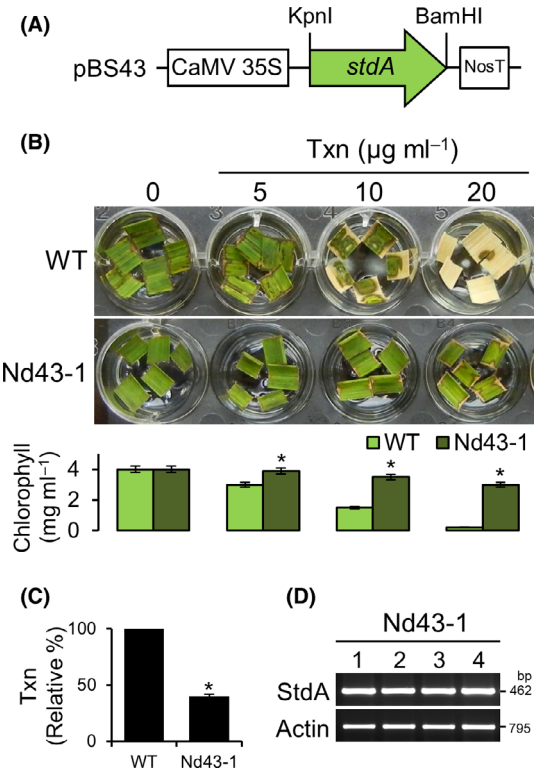

Fig. 6. Transgenic rice plants expressing StdA.
A. Construction of pBS43 (pCAMBIA 1300PT::*stdA*).
B. Leaf disc assay of T_1 rice transgenic line (upper panel). Rice calli were transformed with *A. tumefaciens* GV3101-containing pBS43 or vector. Leaf discs of T_1 rice transformants were immersed in Txn (0–20 μg ml^{-1}), incubated under light (124.5 μmol) and photographed after 4 days. Chlorophyll in the leaf discs (lower panel).
C. Relative percentage of Txn remaining after treatment of rice leaf discs at Txn 10 μg ml^{-1}.
D. Reverse transcriptase-polymerase chain reaction analyses of T_1 rice transformants. Values are averages of duplicate assays; error bars represent the range. Asterisks denote significant differences from the wild type (*$P < 0.05$).

Discussion

Microbial pigments are receiving more attention in current research as they are widely applied as natural food colourants, fires, antimicrobial agents and cytotoxic activity (Ramesh *et al.*, 2019). Yellow Txn exhibits antibiotic activity against a variety of bacteria, fungi and plants and has mammalian toxicity (Latuasan and Berends, 1961; Cox *et al.*, 2000). This toxin is well known as a key virulence factor for bacterial grain rot of rice and wilt of many crop plants (Jeong *et al.*, 2003; Kim *et al.*, 2004). The phytotoxicity of Txn was effective against a broad range of monocot and dicot plants (Koh *et al.*, 2011). Furthermore, Txn has also been shown to demonstrate anticancer activity (Choi et al., 2013a,2013b). These findings led us to investigate the biodegradation and utilization of Txn. In this study, we isolated two bacteria, *S. adhaesiva* SG14 and *Agrobacterium* sp. AL14, with Txn-degrading activity from healthy rice seeds. Surprisingly, even though the two bacteria belong to distinctly different genera, they shared identical nucleotide regions (*stdR* and *stdA*) that function in toxoflavin degradation. In this study, we confirmed that the *stdAR* genes can be transferred by conjugation. These results support the possibility that these bacteria have acquired and shared genes important in antibiotic-Txn degradation through horizontal gene transfer to allow for survival in the same ecological niche. The Txn produced by *B. glumae*, causing rice grain rot, has antibacterial-antifungal activity, so it is difficult for other microorganisms to survive when *B. glumae* is present. We isolated two Txn-degrading bacteria in this study, as well as *P. polymyxa* JH02 with Txn-degrading TflA in a previous study from healthy rice seeds (Koh *et al.*, 2011), and these results suggest that Txn-

resistant bacteria are probably well established in the rice grain environment. In order to complete gene horizontal movement, it is necessary to have components including transposable elements, but there is currently no information on whether the *stdRA* genes are present in these components. It could be interpreted as the result of a war between a rice parasite that use Txn as a weapon and saprophytes trying to overcome it. In addition, it is analysing the extent of matching around two matching genes. Achieved through horizontal gene transfer, the acquisition of antibiotic resistance is necessary for survival in the ecological niche of agricultural animals and humans frequently treated with antibiotics against many bacterial pathogens (O'Brien, 2002). Many variations among closely related bacterial genomes are due to gains and losses of genes that are acquired horizontally as well as to gene duplications and larger amplifications (Francino, 2012). Recently, many findings suggest that specific plasmid might be able to overcome horizontal transfer barriers including host range, phylogenetic and ecological habitat (Zrimec, 2020; Zurfluh *et al*., 2020).

The majority of dioxygenases fully incorporated dioxygen into their substrates, and a variety of cofactor schemes are used to achieve this activity. The most widely observed cofactor involved in dioxygenase reactions is iron. The Txn-degrading enzyme TflA and TxeA require the Mn(II) ion for Txn degradation (Koh *et al*., 2011; Choi *et al*., 2018). Metal ions were not required for the Txn-degrading activity of StdA-His, indicating that StdA has novelty that can be distinguished from many known members of the dioxygenase superfamily. Characterization of StdA mutations trough single amino acid substitutions and high-resolution crystallographic analysis may be required to define the role of specific amino acid residues involved in metal ion independence and Txn binding.

In this study, we found that the expression of *stdA* is regulated by StdR (LTTR family), in the presence of the coinducer, Txn (Fig. 4). These results revealed that the StdR and StdA system operates only in the presence of Txn, so the energy costs for bacteria are efficiently decreased for adaptation to competitive environments to allow for survival in this ecological niche. Because Txn degradation is an energetically expensive process in the absence of Txn, bacteria use complicated regulatory networks to conserve energy and to compete for appropriate niches for survival. There are many other examples of similar regulatory systems, allowing genes to be expressed only when they are needed to utilize or degrade exogenous compounds, such as the use of lactose. In addition, in *S. coelicolor*, the two components VanRS regulate the expression of vancomycin resistance genes only when vancomycin is present (Hutchings *et al*., 2006). In the case of flagella biosynthesis, flagellum genes of bacteria are most likely regulated on multiple levels to conserve energy and to promote the transition between different bacterial developmental phases (Macnab, 1996; Kim *et al*., 2007; Zan *et al*., 2015). Our StdRA regulation was unique compared with a previous report that *tflA*, which encodes a Txn-degrading enzyme, existed independently without a regulator (Koh *et al*., 2011).

It is interesting that StdA is specific for Txn and does not degrade the other derivatives except reumycin without a single methyl group. However, reumycin does not act as a StdR coinducer and consequently does not induce *stdA* expression *in vivo*. The interaction of StdR with the DNA region of the *stdA* promoter is central to the control of *stdA* transcription. Currently, we do not have direct evidence of the interaction between StdR and the *stdA* promoter DNA region. To perform electrophoretic mobility shift assays, the StdR was overexpressed in *E. coli*. Overexpressed StdR was collected in the pellet fraction. We used previously reported methods, such as temperature variation, IPTG concentration, incubation time, Txn induction, sorbitol supplement using betaine and the use of sarkosyl, to improve protein solubility, but StdR remained in the pellet fraction.

The generation of transgenic plants with agriculturally improved features will improve agricultural production and economy. The selection of transgenic plants from wild types is crucial in the development of plant transformation methods. The current antibiotic and herbicide resistance selection systems have many issues, and their uses can be limited. Therefore, a more efficient and safe selection marker system is in high demand. Txn is a known photosensitizer and may be useful as a new selection agent for the generation of transgenic plants, as reported previously (Koh *et al*., 2011). To determine whether transgenic plants expressing *stdA* are resistant to Txn, we transformed *stdA* into rice. Through rice transformation, it was possible to ensure that StdA-expressing rice plants degrade Txn to make it resistant to Txn.

Engineering microbial agents for the biocontrol of plant diseases can provide new ways to use natural microorganisms as microbial inoculums, thereby can be used as an eco-friendly alternative to pesticides (Jing *et al*., 2020). Inactivation of the negatively-acting transcriptional regulator or sensor kinase enhanced the production of antimicrobial secondary metabolites, which contributed to improving the antimicrobial activity to act as a biocontrol agent (Kim *et al*., 2003; Jing *et al*., 2020). In this study, the biological control effect on rice grain rot using Txn-degrading bacteria was not evaluated, so future studies on plant disease control incorporating microbial biotechnology are needed.

In this study, we identified a novel phytotoxin-quenching regulation of bacteria from rice grain, on which phytotoxin-producers co-exist. Our results demonstrate the possibility that if the population of phytotoxin-quenchers were to overwhelm that of producers, the toxicity would be reduced. Manipulating the microbial ecosystem will be an alternative strategy for controlling plant disease. This is the first study to demonstrate that the LysR-type regulator StdR and its coinducer Txn activate expression of the Txn-degrading enzyme StdA. The characterization of StdA may enable the development of disease-resistant crop plants.

Experimental procedures

Bacterial strains and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* strains were cultured on Luria-Bertani (LB) medium at 37°C. *Sphingomonas adhaesiva* SG14 and *Agrobacterium* sp. AL14 were cultivated at 28°C on LB medium or Agrobacterium (AB) minimal medium supplemented with 0.2% glucose. Antibiotics were used at the following concentrations: ampicillin, 50 μg ml^{-1}; kanamycin, 50 μg ml^{-1}; rifampicin, 100 μg ml^{-1}; and gentamycin, 25 μg ml^{-1}. 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) was used at 40 μg ml^{-1} when necessary.

DNA manipulation and sequencing

Standard methods were used for DNA cloning, restriction mapping and gel electrophoresis as described by Sambrook *et al*. (1989). The DNA fragments were sequenced using the BigDye Terminator Kit (Thermo Fisher Scientific, Waltham, MA, USA) with universal and reverse primers. The DNA sequences were analysed using the BLAST program at the National Center for Biotechnology Information (Gish and States, 1993), MEGALIGN (DNASTAR, Madison, WI, USA), and GENETYX-WIN software (Genetyx Co., Tokyo, Japan).

Synthesis of Txn and its derivates

Txn and its derivatives, fervenulin, reumycin, 3-methyltoxoflavin, 3-phenyltoxoflavin, 4,8-dihydrotoxoflavin, 3-methyl 4,8-dihydrotoxoflavin and 6,9-dimethyl-8-oxo-6,9 dihydro-6-azapurine used in this study were synthesized as described by Nagamatsu (2001). Stock solutions of 10 mM in methanol (high-performance liquid chromatography grade) were diluted into the growth medium or working solution to give the stated concentrations.

Table 1. Bacterial strains and plasmids.

Strain or plasmid	Relevant properties	Reference of origin
Sphingomonas adhaesiva		
SG14	Wild type	This study
stdA$^-$	SG14 *stdA*::*lacZY*; Rif^R Km^R	This study
Δ*stdR*	SG14 non-polar deletion mutant of *stdR*; Rif^R	This study
Δ*stdR*/ *stdA*::*lacZY*	SG14 *stdR* non-polar deletion and *stdA*::*lacZY*; Rif^R Km^R	This study
Agrobacterium sp.		
AL14	Wild type	This study
AL14-OK1	*stdA*::Tn*Mar*; Rif^R Km^R	This study
A. tumefaciens		
C58	Wild type	Xu *et al*. (2013)
Escherichia coli		
DH5α/λ*pir*	λ*pir*; cloning strain	Promega
S17-1/λ*pir*	λ*pir*; Tra+, cloning host	Promega
S17-1/ λ*pir* (pFD1)	*Himar*1 conjugal donor	Rubin *et al*. (1999)
BL21(DE3)	F^- *ompT* $hsdS_B(r_B^-\ m_B^-)$ *gal dcm* (DE3)	Novagen
Plasmids		
pGEM-T Easy	PCR cloning vector; Amp^R	Promega
pVIK112	R6K-based *lacZY* transcriptional fusion; Km^R	Kalogeraki and Winans (1997)
pNPTS138-R6KT	*mobRP4*$^+$*ori*-R6K *sacB*; suicide plasmid for in-frame deletions; Km^R	Merrit et al. (2007)
pBBR1-MCS5	Broad host range P_{lac} expression vector; Gm^R	Kovach *et al*. (1995)
pSRKGm	Broad host range P_{lac} expression vector; *lacIq*; Gm^R	Khan *et al*. (2008)
pET21b	T7 promoter-based expression vector; Amp^R	Novagen
pCAMBIA 1300PT (Multi)	Binary vector; Km^R	Cambia
pSK10	pGEM-T Easy carrying internal fragment of *stdA*; Amp^R	This study
pSK12	pVIK112 carrying internal fragment of *stdA*; Km^R	This study
pCOK391	pGEM-T Easy carrying full-length *stdA*; Amp^R	This study
pCOK392	pGEM-T Easy carrying *stdR* SOE deletion fragment; Amp^R	This study
pCOK393	pET21b carrying full-length *stdA*, from pCOK391; Amp^R	This study
pCOK394	pNPTS138-R6KT carrying *stdR* SOE deletion fragment; Km^R	This study
pCOK399	pBBR1-MCS5 carrying full-length P_{lac}–*stdA*; Gm^R	This study
pCOK401	pGEM-T Easy carrying full-length *stdR*; Amp^R	This study
pCOK403	pSRKGm carrying full-length P_{lac} –*stdR*; Gm^R	This study
pCOK423	pET21b carrying full-length *stdR*, from pCOK401; Amp^R	This study
pBS43	pCAMBIA 1300PT(Multi) carrying full-length *stdA*; Km^R	This study

Amp^R, ampicillin resistance; Gm^R, gentamycin resistance; Km^R, kanamycin resistance; Rif^R, rifampicin resistance.

Isolation of Txn-degrading bacteria

To isolate Txn-degrading bacteria, healthy rice seeds were dipped into AB minimal broth containing 40 μg ml^{-1} Txn and incubated for 2 days at 28°C. Colourless AB minimal broth was spread on AB minimal medium containing 40 μg ml^{-1} Txn and bacteria that survived on Txn were selected. To confirm the identity of the bacterial isolates, 16S ribosomal RNA (rRNA) gene sequencing and whole-cell matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) analysis were performed. The 16S rRNA gene polymerase chain reaction (PCR) of the isolates was performed with the primers 27mF (5′-AGAGTTTGA TCMTGGCTCAG-3′) and 1492mR (5′-GGYTACCTT GTTACGACTT-3′). Whole-cell MALDI-TOF MS analysis was performed as described by Seng *et al*. (2009) and was followed by a search using MALDI Biotyper software (ver. 3.0, Bruker Daltonics, Germany) and the reference database.

Transposon mutagenesis, plasmid construction for lacZY*-integration and generation of non-polar deletion mutant*

To construct the transposon mutant, plasmid pFD1 was transferred from SM10λ*pir* pFD1 (*Himar1*) into *S. adhaesiva* SG14 or *Agrobacterium* sp. AL14 by conjugation (Lampe *et al*., 1999; Chiang and Rubin, 2002). Cells were harvested from mating plates and spread on LB containing rifampicin and kanamycin. Surviving cells were spread again on the same media. Chromosomal DNA was prepared from individual mutants (Rubin *et al*., 1999). The insertion site of the mariner-based transposon was analysed by touchdown PCR (Xu *et al*., 2013) with 961_MarTDL2 (5′-GACACGGGCCTCGA NGNNNCNTNGG-3′) and 962_MarRSeq (5′-CGGGTA TCGCTCTTGAAGGGA-3′). PCR products were identified using DNA sequence analysis. The region of 2.1 kb-DNA covering all transposon-directed insertion sites was confirmed by PCR using primers StdB (5′-GGCG GATCCGGAACTCCTGGCAACGAT-3′) and StdH (5′-GGCAAGCTTAAGTTTCCAATAATGCTG-3′) (Fig. 1).

For *lacZY* transcriptional fusion mutagenesis, an internal fragment of *stdA* was amplified with StdAE (5′-G ATCTAGGATTGTCCCAC-3′) and StdAK (5′-GGTA CCAATGCCTATGTGACCGAA-3′). The partial *stdA* fragment was purified and cloned into the pGEM-T Easy vector (Promega, Madison, WI, USA), creating pSK10 and confirmed by sequencing. The *Eco*RI/*Kpn*I-digested *stdA* fragment of pSK10 was cloned into the pVIK112 suicide vector (Kalogeraki and Winans, 1997), creating pSK12. The parent strain SG14 was conjugated with pSK12, and Km-resistant transconjugants were selected. The mutants were confirmed by PCR using a primer that anneals upstream of the truncated fragment and the primer LacFuse (5′-GGGGATGTGCTGCAAGGCG-3′), followed by sequencing.

To generate the non-polar deletion mutant, the outside DNA fragments (approximately 500 bp) of the target gene were amplified. These DNA fragments were upstream (amplified by primers 1 and 2) and downstream (amplified by primers 3 and 4) of the gene to be deleted. Primers 2 and 3 were designed with complementary sequences at the 5′ ends to allow splicing by overlapping extension, as described previously (Merritt *et al*., 2007). The DNA fragments were purified and used as a template for five cycles of PCR. A final PCR was performed with primers 1 and 4 using the short PCR product as a template. The resulting product was cloned into pGEM-T Easy and confirmed by DNA sequencing. The fragment was excised using appropriate restriction enzymes and ligated into the suicide vector pNPTS138-R6KT. Derivatives of pNPTS138-R6KT were introduced into SG14 by conjugation. Single cross-over integrants were selected by growth on LB plates supplemented with rifampicin and kanamycin. Single colonies were grown overnight in LB with rifampicin and spread on LB containing 5% sucrose. Excision of the integrated plasmid was confirmed by patching onto LB supplemented with kanamycin or sucrose. Kanamycin-sensitive colonies were selected and deletion of the targeted DNA was confirmed by diagnostic PCR and DNA sequencing of the product.

Gene complementation

To generate target gene complementary strains, we cloned each intact target gene into broad host range plasmid vectors pBBR1-MCS5 (Kovach *et al*., 1995) or pSRKGm (Khan *et al*., 2008) generating pCOK399 (pBBR1-MCS5::P_{lac}–*stdA*) or pCOK403 (pSRKGm::P_{lac}–*stdR*) and transferred them to corresponding mutant strains by conjugation respectively (Table 1). When necessary, 500 μM IPTG was added.

β-Galactosidase assay

For the β-galactosidase assay, the test strains were grown for 20 h and sub-cultured in LB broth at 28°C. SG14 derivative cultures were supplemented with 1 or 10 μM Txn. Exponential-phase cultures were measured at OD_{600} of ~ 0.4, and β-galactosidase assays were performed immediately. The β-galactosidase activity of the cultures was assayed as described previously (Hibbing and Fuqua, 2011).

Random mutagenesis by error-prone PCR

To generate *stdR* random mutants, *stdR* was amplified using pCOK423 containing the *stdR* region as a

template with primers StdRNd (5′-GGCCATATGCAG GATACGAATAATTTC-3′) and StdRBam2 (5′- GGCG GATCCCTACCTCTGCCCCTCGGGACCGGC-3′), and error-prone PCR product was cloned into pGEM-T Easy, and the mutation site was confirmed by DNA sequencing. The selected error-prone PCR product was digested with *Nde*I and *Bam*HI, and ligated into the corresponding position of isopropyl β-D-1-thiogalactopyranoside (IPTG)-inducible pSRKGm. These plasmids were introduced into the SG14 Δ*stdR stdA*::*lacZY* strain by conjugation. β-Galactosidase assays were performed in SG14 derivatives supplemented with 1 μM Txn and 500 μM IPTG.

Overexpression, purification and enzyme assay of StdA

To overexpress StdA in *E. coli*, the coding region of *stdA* was amplified using the chromosomal DNA of SG14 as the template and the primers StdAN (5′-GGCCATATG ATCCGGTCGTCCGAAACA-3′) and StdAXo (5′-GGC CTCGAGTGTTATAGACGACACTTCGAG-3′), which introduced *Nde*I and *Xho*I sites at the ends of the PCR product; the amplified product was cloned into pET21b (Novagen, Darmstadt, Germany), resulting in pCOK393. StdA-His was overexpressed in *E. coli* BL21(DE3) as described by the manufacturer (Novagen) and purified using a Ni-NTA spin column (Qiagen, Valencia, CA, USA). The eluted protein was dialysed with 50 mM sodium phosphate (pH 8.0) buffer, and the concentration of the purified protein was measured using the Bradford method with bovine serum albumin as the standard (Bradford, 1976).

The enzyme assay of purified StdA-His was performed as described previously, with some modifications (Koh *et al*., 2011). Purified StdA-His was assayed *in vitro* to determine the optimum pH, temperature and dithiothreitol (DTT) requirements to degrade Txn by thin-layer chromatography (TLC). StdA-His activity was assayed at pH 4.5–10 and a temperature range of 15–50°C. The enzyme assays were performed in 50 mM sodium phosphate buffer (pH 8.0). StdA-His (3 μM) in assay buffer (50 mM sodium phosphate buffer pH 8.0, and 5 mM DTT) and 100 μM Txn were incubated for 10 min at 28°C. The enzyme reaction was stopped by the addition of 400 μl of chloroform. The chloroform layer was dried and dissolved in 10 μl of methanol. The methanol extract was developed on a silica gel 60 TLC plate (Merck, Kenilworth, NJ, USA) with chloroform:methanol (95:5, v:v). The degradation of Txn and its derivatives were detected under ultraviolet light (254 or 365 nm). We previously demonstrated that TLC analysis is a simple and easy method for the detection of Txn and its derivatives (Kim *et al*., 2004; Koh *et al*., 2011; Choi et al., 2013a,2013b).

Plasmid construction for plant transformation

The coding region of *stdA* was amplified using the primers, StdAKn (5′-GGCGGTACCATGATCCGGTCGTCC GAAACA-3′) and StdABm (5′-GGCGGATCCTCATGT TATAGACGACACTTC-3′), and the *Kpn*I/*Bam*HI fragment of the PCR product was cloned into pCAMBIA 1300PT(multi), which contains the hygromycin resistance gene under the control of the 35S promoter, resulting in pBS43. This vector was mobilized into *A. tumefaciens* GV3101 by electroporation.

Agrobacterium-*mediated rice transformation*

Mature rice seeds (*Oryza sativa* cv. Nakdong) were dehusked and sterilized. Then the seeds were rinsed with sterile water and dried. For callus induction, sterilized seeds were transferred to NB medium (N6 major salt, B5 minor salt, B5 vitamin, 1× MS_Fe-EDTA, casamino acid 300 mg l^{-1}, proline 500 mg l^{-1}, glutamine 500 mg l^{-1}, sucrose 30 g l^{-1}, 2,4-D 2 mg l^{-1}, phytagel 3 g l^{-1} and incubated for 4 weeks at 25°C in the dark. Embryogenic calli derived from scutella were used for transformation experiments. *Agrobacterium*-mediated rice transformation was performed according to previous methods (Koh *et al*., 2011). The calli were inoculated with *A. tumefaciens* LBA4404 carrying pBS43(pCAMBIA 1300PT::*stdA*) and incubated at 25°C in the dark for 48 h. After co-cultivation, the calli were washed, dried and placed on first selection medium containing 500 mg l^{-1} cefotaxime and 30 mg l^{-1} hygromycin (2N6-CH30 medium) for 2 weeks. After first selection, healthy calli were transferred to the second selection medium containing 500 mg l^{-1} cefotaxime and 50 mg l^{-1} hygromycin (2N6-CH50 medium). Colonies of callus cells that had proliferated on first and second selection medium were transferred on N6-7-CH medium containing 500 mg l^{-1} cefotaxime and 50 mg l^{-1} hygromycin for 10 days at 25°C in the dark. After 10 days, calli have increased in size and showed creamy white colour was shifted onto N6S3-I medium (1/2 N6 major salt, B5 minor salt, 1× MS_Fe-EDTA, B5 vitamin, AA salt and amino acid, casamino acid 2 g l^{-1}, sucrose 20 g l^{-1}, sorbitol 30 g l^{-1}, NAA 1 mg l^{-1}, kinetin 5 mg l^{-1}, cefotaxime 250 mg l^{-1}, phytagel 6 g l^{-1}) for 2 weeks at 25°C in the light conditions (16 h light; 8 h dark). Calli with green small shoot in N6S3-I medium were transferred onto N6S3-II (1/2 N6 major salt, B5 minor salt, 1× MS_Fe-EDTA, B5 vitamin, AA salt and amino acid, casamino acid 2 g l^{-1}, sucrose 30 g l^{-1}, NAA 0.5 mg, Kinetin 2 mg l^{-1}, cefotaxime 250 mg l^{-1}, phytagel 3 g l^{-1}). The shoots differentiated on N6S3-I and N6S3-II medium were transferred to 1/2 MSCH50 medium (1/2 MS salt & vitamin 500 mg l^{-1} cefotaxime and 50 mg l^{-1}

hygromycin) for rooting and final selection. Transgenic rice plants (T_0 generation) were eventually transferred to soil in pot and grown in greenhouse.

Molecular analysis of putative transgenic rice plant

The relative quantity of *toxin* transcript in transgenic rice plants was analysed by reverse transcriptase-PCR (RT-PCR). Total RNAs were extracted from T_1 transgenic and wild-type rice plants using Trizol according to the manufacturer's instruction (Invitrogen). The cDNAs were synthesized from 2 μg of total RNA using RevertAid™ First Strand cDNA Synthesis Kit (Fermentas) in 20 μl reaction volume at 42°C for 60 min. Reverse transcription of all the RNA samples was carried out using oligo $(dT)_{18}$ primer. Rice *actin* gene was used as an endogenous control in RT-PCR using the primer ACTIN-forward (5'-GGAACTGGTATGGTCAAGGC-3') and ACTIN-reverse (5'-AGTCTCATGGATACCCGCAG-3'). The toxin gene was amplified using the primers, StdAKn and StdABm and for rice actin gene were 5'-GTAAGCAAC TGGGATGACATGGAGAA-3' and 5'-CCTCCAATCCA GACACTGTACTTCCTC-3'. RT-PCR was performed at 94°C for 2 min for initial denaturation followed by 30 amplification cycles comprising of 30-sec denaturation at 94°C, 30-sec annealing at 55°C, 30 s extension at 72°C in 20 μl reaction mixture using laboratory taq polymerase. PCR products were analysed by electrophoresis in 1% agarose gel.

Conjugation assays

For conjugation assays, kanamycin-resistant *stdA*::*lacZY* mutant strains of SG14 or AL14 were used as donor strains. As a recipient, gentamycin-resistant *A. tumefaciens* C58 strain harbouring pSRKGm was used. Conjugation assays were performed by mixing suspensions of donor and recipient cells according to the previous report (Heckel *et al*., 2014). Each strain was normalized to an OD_{600} of 0.8 and 100 μl-suspension of donor and recipient cells were spotted onto cellulose acetate filters on AB minimal medium, and the mating plates were incubated at 28°C for 24 h. The cellulose membrane was harvested and suspended in 500 μl sterile normal saline. The mating suspension was spread on AB minimal medium supplemented with 150 μg ml^{-1} kanamycin and 300 μg ml^{-1} gentamycin. Conjugation efficiency was determined as the ratio of transconjugants per output donor. All transconjugants confirmed the presence of the *stdA* and *virD* genes by multiplex PCR amplification. The primers used for the detection of *stdA* and *virD* genes were StdAE, LacFuse, virD2A (5′-ATGCCCGATCGAG CTCAAGT-3′) and virD2C (5′-TCGTCTGGCTGACTTT CGTCATAA-3′).

Leaf disc assays and chlorophyll quantification

Rice leaf disc assays (Koh *et al*., 2011) and chlorophyll quantification assays (Amon, 1949) were performed as described previously. The Txn degradation assay by rice plants was performed similarly the rice leaf disc assay. Ten rice leaf discs (5 × 5 mm) were placed in sterilized water containing 10 μg ml^{-1} Txn, and after 48h, the remaining Txn was quantified according to the previous report (Kim *et al*., 2004).

Conclusion

Toxoflavin (Txn), broad host range phytotoxin produced by a variety of bacteria, is a key pathogenicity factor of *Burkholderia glumae* in rice and field crops. Two bacteria exhibiting Txn-degrading activity were identified as *Sphingomonas adhaesiva* and *Agrobacterium* sp. respectively. The genes *stdR* and *stdA*, which are responsible for Txn degradation of both bacterial isolates, were identical, indicating that horizontal gene transfer occurred between microbial communities in the same ecosystem. We identified a novel phytotoxin-quenching regulation of bacteria, demonstrating that the transcriptional regulator StdR positively regulates expression of the *stdA* gene, which encodes a Txn-degrading enzyme, in the presence of Txn as a coinducer. Amino acid sequence of StdA exhibited similarity with a metalloenzyme, but metal ions were not required for Txn degradation. Rice plants expressing StdA showed Txn resistance.

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Conflicts of interest

The authors declare no conflict of interest.

Author contributions

OC and JK conceived and designed the project. OC, YL, BK and JP performed experimental work. HJC and MCK

performed rice plant assays. OC and JK wrote the manuscript with assistance and input of coauthors.

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Supporting information

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

Fig. S1. Alignment of amino acid sequences of StdA with other dioxygenases. Amino acid sequences of StdA were aligned with the Txn resistance protein ToxM from *Pseudomonas protegens* Pf-5 (WP_011059383), Txn-degrading enzyme TflA from *Paenibacillus polymyxa* JH2 (ADK47414), glyoxalase from *Exiguobacterium sibiricum* (WP_012370233), CatE from *Bacillus halodurans* (WP_010898299), HpaD from *Geobacillus genomosp. 3* (BAD08313), ThnC from *Sphingopyxis macrogoltabida* (AF157565), and BphC from *Paraburkholderia xenovorans* LB400 (ABE37053). The conserved residues (>50%) of all eight sequences are boxed. The sequence alignment was performed using DNAMAN (Lynnon Co., Quebec, Canada).

Fig. S2. Characterisation and overexpression of StdA. (A), Purification of the StdA–His protein. Overexpression of His-tagged StdA protein in *E. coli* BL21(DE3) was induced by 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and purified by nickel-nitrilotriacetic acid (Ni-NTA) column by washing with imidazole. Purified StdA–His is shown in a Coomassie blue-stained polyacrylamide gel (PAGE). M, molecular mass standards; lane 1, total cell extraction of BL21(DE3) harbouring pCOK393 following 1 mM IPTG induction; lane 2, insoluble fraction; lane 3, soluble fraction; lane 4, flow through; lane 5, wash with 20 mM imidazole; and lanes 6–8, final elutions of 1, 5 and 10 μl StdA–His respectively. Separation was performed using 10% sodium dodecyl sulphate PAGE. Bands were visualized after staining with Coomassie blue. The band corresponding to StdA–His is indicated with an arrow. (B), Txn degradation activity of purified StdA–His at various temperatures. T, no StdA–His; lane 2, 5–80°C. StdA–His (3 μM) in assay buffer 50 mM sodium phosphate buffer pH 8.0, 5 mM DTT and 100 μM Txn were incubated for 10 min.

Fig. S3. Chemical structures of Txn and its derivatives used in this study. Txn and its derivatives, fervenulin, reumycin, 3-methyltoxoflavin, 3-phenyltoxoflavin, 4,8-dihydrotoxoflavin, 3-methyl 4,8-dihydrotoxoflavin and 6,9-dimethyl-8-oxo-6,9 dihydro-6-azapurine used in this study were synthesized as described by Nagamatsu (2001). Stock solutions of 10 mM in methanol (high-performance liquid chromatography grade) were diluted into the growth medium or working solution to give the stated concentrations.

Fig. S4. Multiplex PCR for simultaneous detection of *stdA* and *virD* genes in the C58(pSRKGm) transconjugants conjugated with the donor strains SG14 *stdA*::*lacZY* (upper panel) and AL14 *stdA*::*lacZY* (lower panel). M, DNA size marker; D, donor; R, recipient; and 1–5, five C58(pSRKGm) transconjugants.

