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A simple and sensitive biosensor strain for detecting toxoflavin using β-galactosidase activity

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ABSTRACT

In this study, we developed a simple and sensitive biosensor for the determination of toxoflavin (which is toxic to various plants, fungi, animals, and bacteria) in natural samples based on β-galactosidase activity. The proposed toxoflavin detection method for toxin-producing bacteria or toxin-contaminated foods is simple and cost effective. *Burkholderia glumae*, a species known to cause rice grain rot and wilt in various field crops, produces toxoflavin under the control of a LysR-type transcriptional regulator ToxR and its ligand toxoflavin. As the expression of toxoflavin biosynthetic genes requires toxoflavin as a co-activator of ToxR, a novel biosensor stain was constructed based on *lacZ* reporter gene integration into the first gene of the toxoflavin biosynthesis operon, *toxABCDE* of *B. glumae*. The biosensor was composed of a sensor strain (COK71), substrates (X-gal or ONPG), and culture medium, without any complex preparation process. We demonstrated that the biosensor strain is highly specific to toxoflavin, and can quantify relative amounts of toxoflavin compared with known concentrations of toxoflavin. The proposed method was reliable and simple; samples containing 50–500 nM of toxoflavin could be analyzed. More importantly, the proposed biosensor strain could identify toxoflavin-producing bacteria in real samples. The excellent performance of this biosensor is useful for diagnostic purposes, such as detecting toxoflavin-contaminated foods and environmental samples.

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1. Introduction

Toxoflavin is the prosthetic group of the yellow pigment, which is produced by *Bacterium bongkrek* (Van Veen and Mertens, 1934). Toxoflavin {1,6-dimethylpyrimido[5,4-*e*]-1,2,4-triazine-5,7(1*H*,6*H*)-dione: molecular weight=193} is an azapteridine toxic to various plants, fungi, animals, and bacteria. It is produced by several bacterial species, including *Burkholderia glumae*, *Burkholderia gladioli*, *Burkholderia plantarii*, and *Streptomyces* (Levenberg and Linton, 1966; Lynch and Dennis, 2010).

Toxoflavin is essential for the pathogenicity of rice seedling and grain rot (also called bacterial panicle blight) of *B. glumae*, probably owing to its mode of action in plants. In *B. glumae*, toxoflavin production is cell-density dependent and under the control of a cell-to-cell communication system called quorum sensing (QS). QS regulates multiple phenotypes in *B. glumae*, including the biosynthesis of bright yellow toxoflavin production, flagellar biosynthesis, and stress responses to visible light or heat shock (Chun et al., 2009; Kim et al., 2004, 2007, 2009, 2012). The QS circuit in *B. glumae* BGR1 consists of the TofI protein, which synthesizes C8-HSL, and its cognate receptor TofR. The C8-HSL and TofR complex induces expression of the toxoflavin biosynthetic operon (*toxA–E*) by activating the transcription of ToxJ, which is required for the expression of both *tox* operons (Kim et al., 2004, 2009). In addition to QS and ToxJ, ToxR is required for the expression of *tox* operons in the presence of toxoflavin as a coinducer. Mutations in ToxR reduce the transcription of *toxA–E* biosynthetic operons, inhibiting toxoflavin production (Kim et al., 2004, 2009). Toxoflavin is thought to function as an electron carrier and generate hydrogen peroxide, bypassing the cytochrome system in the presence of oxygen and light (Latuasan and Berends, 1961; Nagamatsu, 2001). Given the light-dependent generation of hydrogen peroxide (which has high cell toxicity), a novel selection marker system was developed by introducing the *tflA* gene, encoding toxoflavin degradation enzyme, into plants (Koh et al., 2011).

Current outbreaks of bacterial grain and sheath rot of rice caused by *Burkholderia* in rice-growing countries pose a potential threat to food production. As no selective medium currently exists, highly sensitive and specific molecular and biological techniques that can detect toxoflavin during outbreaks of food intoxication

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are important. Simple detection methods for toxoflavin-producing bacteria or toxoflavin-contaminated foods should be as simple and cost effective as possible because these infections occur in economically poor urban regions of many rice-grain-producing countries (Lynch and Dennis, 2010). An effective method for detecting and quantifying the toxoflavin concentrations produced by bacteria is high-pressure liquid chromatography (HPLC) (Voragen et al., 1982). However, the HPLC system requires extraction of toxoflavin from the sample prior to analysis as well as specialized equipment, which can be very expensive.

Therefore, the aim of this study was to utilize current knowledge of toxoflavin regulation in *B. glumae* BGR1 to construct a simple visual toxoflavin biosensor. The biosensor strain can be used to screen bacterial cultures for toxoflavin, to quantify the amounts of toxoflavin produced, and as an analytical and preparative aid in determining the presence of this toxic molecule. Measurement of toxoflavin concentrations based on chemical methods is only possible using pure toxin samples. Since methods based on biological activity have been used to quantify toxoflavin, we developed a simple and highly specific biological procedure to estimate the concentration of toxoflavin within diseased grains of rice and other toxin-contaminated samples.

2. Materials and methods

2.1. Strains, plasmids, and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 1. The *Escherichia coli* strains used for plasmid DNA transformation or conjugational transfer of plasmids were grown in Luria Bertani (LB) medium with or without 1.5% (w/v) agar. Unless otherwise noted, *B. glumae* strains were grown in LB. Chemicals, antibiotics, and culture media were obtained from Fisher Scientific and Sigma-Aldrich. When required, appropriate antibiotics were added to the medium as follows: for *E. coli*, 50 μg/ml ampicillin and 50 μg/ml kanamycin (Km). Additionally, 40 mg/ml of 5-bromo-4-chloro-indolyl-β-D-galactopyranoside (X-Gal) was used when necessary.

2.2. General DNA manipulation

Isolation of plasmid and genomic DNA and transformations were performed according to the procedures described by Sambrook and Russell (2001), and DNA sequencing was performed using an ABI 3730 sequencer. The restriction and DNA-modifying enzymes (New England Biolabs) were used according to the manufacturer's recommendations. DNA fragments were purified from agarose gels using the QIAEX II gel extraction kit as described by the manufacturer (Qiagen). PCR amplification of the DNA was performed using an Astec PC 802 thermal cycler (Astec, Fukuoka, Japan) and standard protocols.

2.3. Construction of chromosomal lacZ transcriptional fusions

Plasmid pCOK71 carrying the internal fragment of the *toxA* gene was constructed using BGR1 genomic DNA as the PCR template and ToxAE (5′-GAATTCCACGTGCGCGACGTG-3′) and ToxAK (5′-GGTACCAAAATCCGGGTCGACGGTATA-3′) as primers. The amplified region (180 bp) was purified from agarose gel, ligated into pGEM-T Easy vector (Promega), confirmed by sequencing, and ligated with the suicide vector pVIK112 (Kalogeraki and Winans, 1997) after restriction digestion with EcoRI and KpnI. The resulting construct, pCOK71, which cannot be replicated by *B. glumae*, was transformed into *E. coli* S17-1 λ*pir*, transferred into *B. glumae* BGR1 by conjugation, and marker-exchanged, to construct COK71. The insertion mutant was selected based on the kanamycin-resistance phenotype and confirmed with PCR and sequence analyses.

2.4. Synthesis of toxoflavin, fervenulin, reumycin, and their analogs

Toxoflavin, fervenulin, reumycin, and their analogs (3-methyltoxoflavin, 3-phenyltoxoflavin, 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 (Kim et al., 2009; Nagamatsu, 2001). Stock solutions (10 mM) in methanol (high-performance liquid chromatography grade) were diluted into growth medium to achieve the stated concentrations.

2.5. Culture plate assays

For plate assays, medium containing the biosensor cells and X-gal was poured into culture plates. When the agar solidified, the strains to be tested for toxoflavin production were spotted directly onto the surface of the plates and incubated overnight at 37 °C.

Toxoflavin production was also assayed by cross-streaking against the toxoflavin biosensor. Briefly, COK71 was streaked on

Table 1
Bacterial strains and plasmids used in this study.

Strain or plasmid	Description[a]	Source or reference
E. coli strain		
DH5α	Cloning host	Gibco-BRL
S17-1 λ*pir*	Cloning host	Simon et al. (1983)
B. glumae strain		
BGR1	Wild-type, Rif^R	Jeong et al. (2003)
BGK59	BGR1 *toxA*::Tn*3-gusA59*	Kim et al. (2004)
COK71	BGR1 *toxA–lacZ* transcriptional fusion, Km^R	This study
B. gladioli strain		
BSR3	Wild-type	Seo et al. (2011a, 2011b)
P. ananatis strain		
PA13	Wild-type	Choi et al. (2012a, 2012b)
Plasmids		
pGEM-T Easy	PCR cloning vector; Amp^R	Promega
pVIK112	R6K suicide vector, *lacZY* for transcriptional fusion, Km^R	Kalogeraki and Winans (1997)
pCOK71	pVIK112, *toxA* internal fragment, transcriptional fusion, Km^R	This study

[a] Amp^R, ampicillin resistance; Km^R, kanamycin resistance; Rif^R, rifampicin resistance.

the center of the LB agar plate containing X-gal. The target bacteria were streaked on the same plate next to the COK71 line. The assay was based on the diffusible toxoflavin produced by the target bacteria, which induces β-galactosidase production in strain COK71.

2.6. Extraction of culture supernatants and analytical TLC

Extracts for analytical TLC and bacterial strains were inoculated into 5 ml of LB broth for 24 h with shaking. Each 0.5 ml of culture was centrifuged at 10,000 × *g* to remove bacterial cells, supernatants were extracted with equal volumes of chloroform, and combined extracts were evaporated to dryness. After evaporation, the residual material was dissolved in 10 μl of methanol.

For analytical TLC, samples (1–4 μl) were applied to silica gel 60 TLC plates (Merck), and chromatograms were developed with chloroform/methanol (95:5, v/v). After development, the solvent was evaporated, and the dried plates were overlaid with soft agar containing *B. glumae* COK71 toxoflavin indicator. A portion of the 1-ml overnight culture of the bacterial indicator cells and X-gal were used to inoculate 50 ml of LB. After the agar solidified, the overlaid plates were incubated at 37 °C in a closed plastic container.

2.7. β-Galactosidase activity assays

β-Galactosidase activity assays were performed in triplicate at least three times, as described previously (Miller, 1972). Mid-log phase biosensor strain COK71 cultures were diluted 1:100 dilution to an OD_{600} of ~0.01 and were supplemented with different concentrations of toxoflavin or toxoflavin derivatives. The cell suspension was thoroughly mixed and aliquoted into 15-ml test tubes, and incubated at 37 °C for 12 h to an OD_{600} of ~0.6. Mid-log phase cultures were measured at OD_{600} and used for subsequent β-galactosidase assays.

2.8. Rice samples and crude extract preparation

Diseased rice panicles were collected in the Jinju area of South Korea from July to October 2011. All samples were kept in a cold room until analysis. Rice kernels (2.5 g) were sampled and mixed with 30 ml chloroform and left for 10 min at room temperature. The solution was blended at 8000 rpm for 5 min and centrifuged at 3000 rpm. After the supernatants were filtered through Whatman Grade no. 3 filter paper, the filtrates were evaporated, after which the chloroform residue was dissolved in 0.5 ml of methanol. We used 0, 5, 10, 15, and 20 μl of sample extracts in methanol for β-galactosidase activity assays. Toxoflavin (100 nM) was used as a positive control.

3. Results and discussion

3.1. Construction of the toxoflavin biosensor strain

Toxoflavin production and regulation circuits in *B. glumae* BGR1 are schematically depicted in Fig. 1. The *toxA* gene is controlled in response to toxoflavin by the transcriptional activator ToxR. We used this activation system to induce *toxA* expression, which allows for toxoflavin controlled expression. The internal fragment of the *toxA* gene was cloned into pVIK112 to measure β-galactosidase activity in response to toxoflavin concentrations. The plasmids were then transferred from S17-1/*λpir* into *B. glumae* strain BGR1 by conjugation, selecting for the kanamycin-resistant *toxA–lacZ* transcriptional fusion. The ToxA null mutation abolished toxoflavin production.

3.2. Responses of COK71 biosensor strain to exogenous toxoflavin

The first step in determining whether the *toxA–lacZ* transcriptional fusion responds to exogenous toxoflavin was to test for β-galactosidase activity. The biosensor strain developed a blue color on X-gal agar in the presence of toxoflavin. No phenotypic changes were observed in the absence of toxoflavin. The toxoflavin biosensor strain could be used in different ways; first, the tester strain could be streaked and grown on solid media close to the biosensor to form a "T" shape, and the phenotypic change associated with the presence of exogenous toxoflavin could be observed as a gradient, with the strongest response observed at the meeting point of the two strains. *B. glumae* BGR1 was streaked and grown on LB media containing X-gal against *B. glumae* COK71 as the toxoflavin biosensor to form a T-streaking. As BGR1 stimulated the blue-colored phenotype of the COK71 biosensor, COK71 could respond to toxoflavin (Fig. 2a).

Second, the tester strain could be spotted onto the medium containing the agar suspension of the toxoflavin biosensor strain, and the phenotypic change associated with toxoflavin production could be observed as a ring with responses observed around the tester strains. *B. glumae* BGR1, *B. gladioli* BSR3, and *Pantoea ananatis* PA13 were spotted onto the LB containing X-gal and suspension of the biosensor strain COK71. As BGR1 and BSR3 caused a blue-colored ring to form around the colonies, BGR1 and BSR3 produced toxoflavin. No phenotypic changes were observed around the toxoflavin non-producer, PA13. This suggested that COK71 responded to toxoflavin (Fig. 2b).

Toxoflavin is not restricted to *B. glumae*, and is also produced by *Streptomyces*, *B. plantarii*, *B. gladioli* pv. *cocovenenans*, and phytopathogenic *B. gladioli* (Levenberg and Linton, 1966; Lynch and Dennis, 2010). Intoxication may occur as a result of eating foods contaminated with toxoflavin-producing bacteria. In mice, the LD_{50} for the ingested toxin is 8.4 mg/kg (Lijmbach et al., 1970).

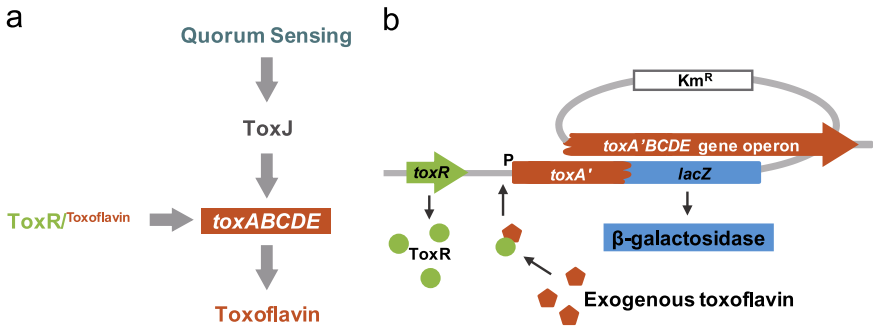

Fig. 1. The toxoflavin production and regulatory circuits of *Burkholderia glumae* (a). ToxJ is controlled by quorum sensing and activates *tox* operon transcription. ToxR is also required to activate the *tox* operon with toxoflavin as a coinducer. Schematic principle of the biosensor strain COK71 for toxoflavin detection (b). Arrows signify positive regulatory interactions. β-Galactosidase enzyme activity represents the presence of exogenous toxoflavin.

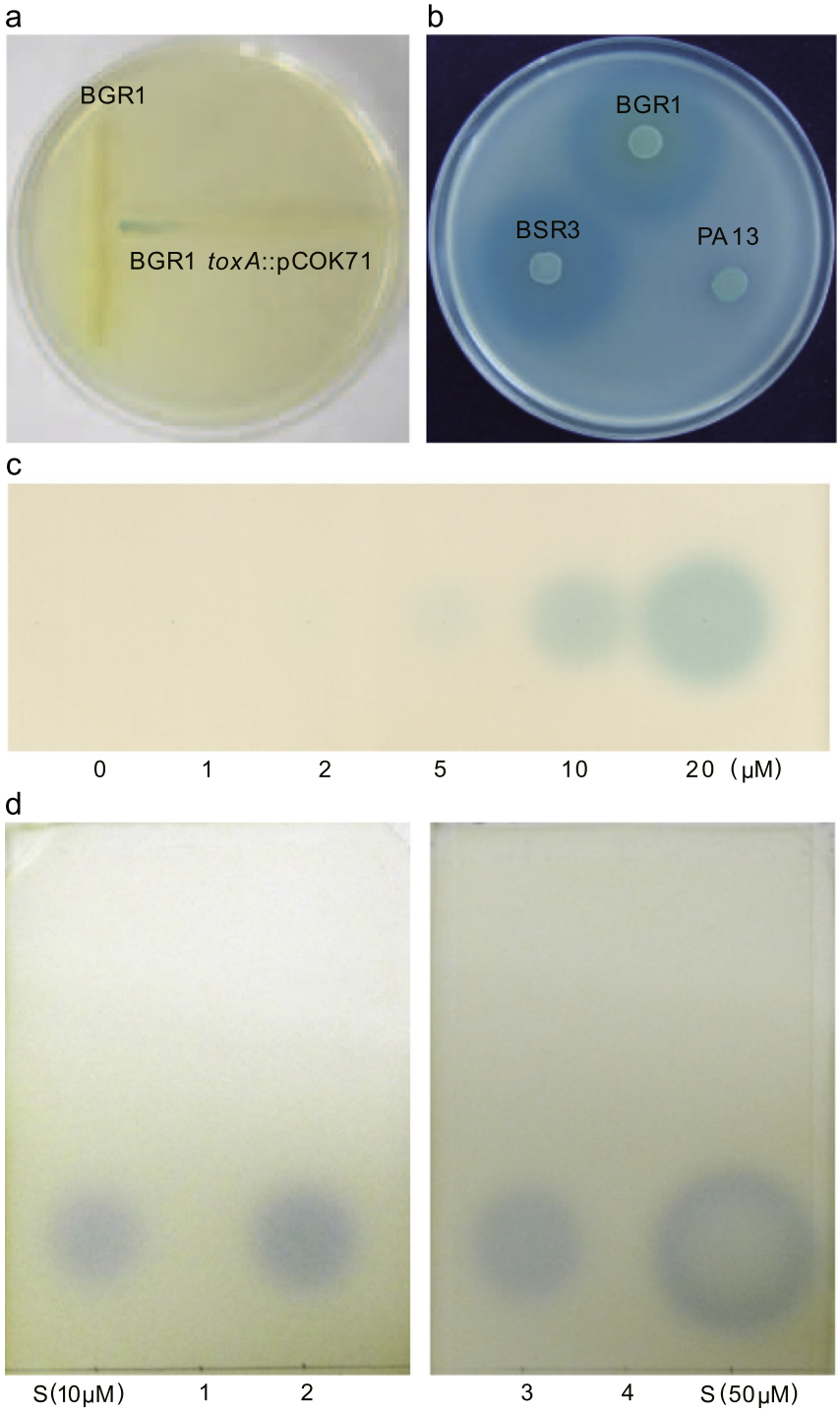

Fig. 2. Detection of toxoflavin using the bacterial biosensor strain. (a) Cross-streaks of BGR1 against toxoflavin biosensor strain COK71. (b) Spotting of tester strains onto LB medium containing agar suspension of the biosensor strain COK71 with X-gal. Diffusible toxoflavin production by BGR1 induced the COK71 biosensor to produce β-galactosidase. (c) TLC analysis of toxoflavin. (d) TLC analysis of toxoflavin produced by strains BGR1 and BSR3. Toxoflavin appeared as a blue-colored ring by COK71 biosensor and was identified after comparison with the synthetic toxoflavin standard. 1, *B. glumae* strain *toxA*::Tn*3-gusA59*; 2, *B. glumae* strain BGR1; 3, *B. gladioli* strain BSR3; 4, *P. ananatis* strain PA13; S, synthetic toxoflavin standard. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Therefore, the proposed biosensor strain could be used to identify toxoflavin-producing bacteria.

Third, toxoflavin could be extracted from spent supernatants of late exponential phase cultures, and partial characterization could be achieved through separation on TLC silica plates. Toxoflavin was generally dissociated into the organic phase, and the solvent was removed by drying. The TLC plates were loaded with sample extracts and, after chromatography, were overlaid with a soft-agar suspension of the toxoflavin biosensor strain. Toxoflavin migrated with a characteristic mobility and resulted in a unique response spot shape that was detected depending on the *lacZ* reporter of the biosensor strain. Separation using TLC coupled with detection by a toxoflavin biosensor provided a rapid and direct visual index of toxoflavin. To determine the detection limit of toxoflavin on TLC, varying amounts of toxoflavin were spotted and a toxoflavin biosensor COK71 was overlaid on the TLC. Increasing amounts of toxoflavin increased the size of induced

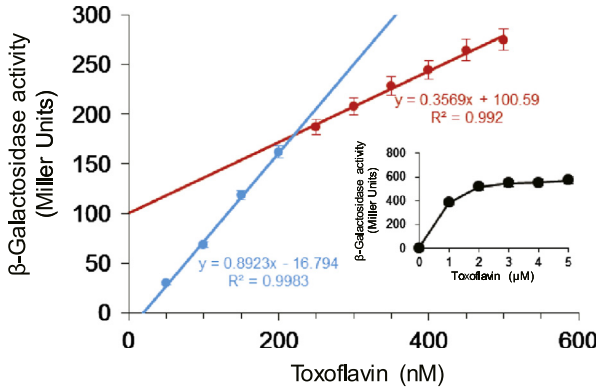

Fig. 3. Plot of the influence coefficient of β-galactosidase activity *versus* the toxoflavin concentration of the proposed biosensor. The results exhibited a good linear relationship in the ranges of 50–200 nM (blue) and 250–500 nM (red) with different slopes. Error bars represent the standard deviations of three independent measurements. The biosensor was grown in LB medium with various concentrations of synthetic toxoflavin. After 5 h of incubation, β-galactosidase activity was measured. The results were reproduced in three repeated experiments, and error bars represent standard deviations. The inset denotes the influence of toxoflavin on *toxA–lacZ* expression, as described above. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

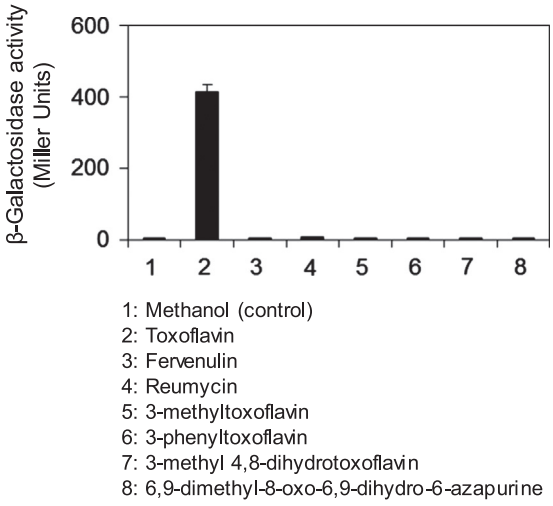

Fig. 4. Expression of *toxA* regulated by ToxR and the coinducer toxoflavin or its derivatives measured in the biosensor strain COK71.

zones surrounding the spots for the toxoflavin biosensor strain COK71. The limit of detection for toxoflavin in the TLC assay was determined to be 5–10 μM (Fig. 2c). As BGR1 and BSR3 both induced blue-colored rings on TLC, they produced toxoflavin. Phenotypic changes were not observed for the toxoflavin non-producer, PA13, on TLC (Fig. 2d). Our data suggest that toxoflavin biosensor strain COK71 could be as effective as a simple visual biosensor for toxoflavin detection.

Diffusible responses of toxoflavin to the biosensor strain COK71 suggested that toxoflavin (regulated by QS) alters gene expression patterns. Recently, experimental support for this hypothesis was established using *Pseudomonas aeruginosa* and *Pseudomonas chlororaphis* (Dietrich et al., 2008). In both Pseudomonads, phenazine modulated their physiology, and several genes were up- or down-regulated by phenazine production (Pierson and Pierson, 2010). Thus, phenazines can serve as signals in both *Pseudomonas* species. These results suggested that the diffusibility of toxoflavin may affect the expression of several gene sets as a signal. However, further studies are required to address this hypothesis.

3.3. Detection of toxoflavin based on β-galactosidase activity assays

The toxoflavin biosensor could also be used to quantify toxoflavin by measuring the activity of the *lacZ* reporter system in the biosensor bacterial strain. To perform an accurate quantification using synthetic toxoflavin, the minimal amount of toxoflavin required to generate a response, as well as the amount necessary for a saturated response, must be determined. Thus, the linear dose response should be calculated. We assayed toxoflavin for the ability to induce β-galactosidase activity in strain COK71, which carries *toxA–lacZ* transcriptional fusion. Under the optimal experimental conditions established in the above studies, calibration plots were generated for toxoflavin (Fig. 3). The results exhibited a good linear relationship in the ranges of 50–200 nM and 250–500 nM with different slopes, and the slope of the low concentration (50–200 nM) was greater than that of the high concentration (250–500 nM). Such situation of β-galactosidase enzyme activity was also observed by several other groups (Sambrook and Russell, 2001; Danhorn et al., 2004). Synthetic toxoflavin activated this fusion at a concentration of 50 nM (Fig. 3). A concentration of 127 nM generated approximately 100 U of enzyme activity. Within a toxoflavin range of 1 to 5 μM, enzyme activity increased from 385 to 576 U (Fig. 3, inset).

The proposed method showed good sensitivity for toxoflavin detection. The detectable amount of toxoflavin in the β-galactosidase activity assays was found to be 50 nM, and was 100-fold higher than that of TLC analysis. Chemical extraction and determination of toxoflavin using the HPLC system was described by Voragen et al. (1982). HPLC has previously been used to detect toxoflavin concentrations from 1 to 300 μg/ml (5.17 to 1551 μM) (Voragen et al., 1982). The detectable amount of toxoflavin in the proposed biosensor was 100-fold greater than that using HPLC analysis. In mice, the LD_{50} for injected toxoflavin is 1.7 mg/kg (Lijmbach et al., 1970). Thus, the limit of detection of the proposed method is enough to satisfy the LD_{50}, and the sensitivity of the proposed method is acceptable. Overall, this system effectively quantifies and detects toxoflavin levels.

3.4. Specificity of the biosensor to toxoflavin and its derivatives

As many biosensors rely on a particular ligand-binding protein, they display specificity towards the cognate ligand. Additionally, as many biosensors are capable of detecting only a narrow range of ligands, it is essential to use several ligands when testing a bacterium for ligand production. To determine whether strain COK71 can be used as a biosensor to detect toxoflavin, we tested toxoflavin, fervenulin, reumycin, and their derivatives by evaluating the expression levels of *toxA* in the biosensor strain COK71 (Fig. 4). The natural *B. glumae* ligand, toxoflavin, exhibited the strongest reaction using this assay, activating *toxA* expression at a concentration of 1 μM. In contrast to toxoflavin, its derivatives did not activate *toxA* expression. No response to other derivatives was achieved at a concentration of even 10 μM, reflecting the high degree of specificity of this ToxR transcriptional regulator for its cognate ligand, toxoflavin (Fig. 4). This result reflects a general trend in which the *Burkholderia* COK71 *tox* system responds much more strongly to toxoflavin than to other derivatives. Transcription of the *toxA* gene in response to toxoflavin very closely matched toxoflavin production (Fig. 4). These data suggest that strain COK71 is highly specific for toxoflavin detection and agrees with previous studies of *toxA* expression using *gusA* reporter and toxoflavin derivatives (Kim et al., 2009).

3.5. Detection of toxoflavin in rice panicle samples

Rice grain rot and palea browning symptoms are often found in rice-growing regions. Additionally, rice grain discoloration caused by *Pantoea* spp. is often mistaken for rice grain rot. Diseased rice

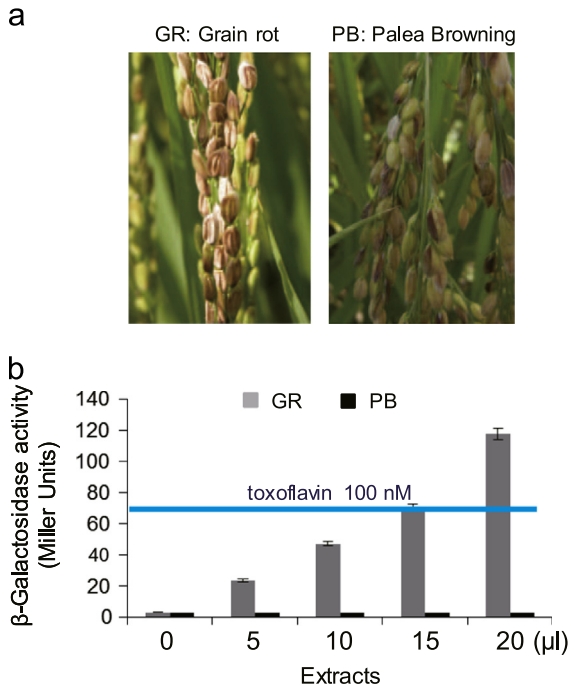

Fig. 5. (a) Natural symptoms of bacterial grain rot or palea browning of rice. (b) Detection of toxoflavin produced *in vivo*. Induction of the *toxA–lacZ* transcriptional fusion in toxoflavin biosensor strain COK71. The biosensor was grown in LB medium with chloroform extracts from diseased rice grains (see Material and methods). After 5 h of incubation, β-galactosidase activity was measured. The results were reproduced in three repeated experiments, and error bars represent standard deviations. GR, grain rot; PB, palea browing.

kernels were sampled from the paddy fields in the summers of 2010 and 2011. Each sample was divided into two distinct symptoms; namely, grain rot and palea browning (Fig. 5a). Chloroform was used to extract toxoflavin from diseased rice kernels. To determine whether the biosensor for toxoflavin detection from contaminated natural samples worked effectively, the β-galactosidase activity of strain COK71 was measured in a liquid medium containing crude chloroform extracts from diseased rice kernels. Only the crude extract sampled from rice grain rot exhibited β-galactosidase activity, whereas extracts sampled from healthy or palea browning symptoms did not activate β-galactosidase activity (Fig. 5b). This suggested that *B. gluame* produces toxoflavin *in vivo*. Application of 15 μl methanol residue from the chloroform extract induced 70 U of β-galactosidase activity and 100 nM toxoflavin induced 69 U of β-galactosidase activity. The dose–response curve shown in Fig. 5 suggests that 70 U of enzyme activity would indicate a concentration of 97 nM toxoflavin (Fig. 5b). This dose–response curve was used to estimate toxoflavin doses as 251 μg per kilogram in diseased grain rot samples. These data suggest that toxoflavin biosensor strain COK71 could be applied to estimate of the concentration of toxoflavin within diseased rice grains or toxin-contaminated samples. As a result, the proposed method can efficiently detect toxoflavin in rice samples.

4. Conclusions

In this study, a novel biosensor stain was constructed based on *lacZ* reporter gene integration into the first gene of the toxoflavin biosynthesis operon, *toxABCDE* of *B. glumae*. The biosensor was composed of a sensor strain (COK71), substrates (X-gal or ONPG), and culture medium, without any complex preparation process. Using the biosensor strain, we detected toxoflavin in natural samples. The proposed biosensor strain described here offers many advantages, such as easy sample preparation, simple detection processes, high sensitivity and specificity, and low cost for toxoflavin detection. This biosensor strain can be applied to detect or quantify toxoflavin concentrations. Additionally, it could be used to detect toxoflavin within plant leaves, grains, soils, and environmental samples.

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