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Novel biosensors based on flavonoid-responsive transcriptional regulators introduced into *Escherichia coli* ☆

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ABSTRACT

This study describes the construction of two flavonoid biosensors, which can be applied for metabolic engineering of *Escherichia coli* strains. The biosensors are based on transcriptional regulators combined with autofluorescent proteins. The transcriptional activator FdeR from *Herbaspirillum seropedicae* SmR1 responds to naringenin, while the repressor QdoR from *Bacillus subtilis* is inactivated by quercetin and kaempferol. Both biosensors showed over a 7-fold increase of the fluorescent signal after addition of their specific effectors, and a linear correlation between the fluorescence intensity and externally added flavonoid concentration. The QdoR-biosensor was successfully applied for detection of kaempferol production *in vivo* at the single cell level by fluorescence-activated cell sorting. Furthermore, the amount of kaempferol produced highly correlated with the specific fluorescence of *E. coli* cells containing a flavonol synthase from *Arabidopsis thaliana* (*fls1*). We expect the designed biosensors to be applied for isolation of genes involved in flavonoid biosynthetic pathways.

1. Introduction

Plant secondary metabolites are an important source of new drugs and nutraceuticals. Phenylpropanoids, especially flavonoids and stilbenes, were shown to have antioxidant, antiviral, antibacterial, anticancer and immunosuppressive activities (Clere et al., 2011; Gresele et al., 2011; Pan et al., 2010). The commercial availability of these compounds is mainly limited by two factors: (i) generally present in low intracellular concentrations in the complex host cell matrix and (ii) high chemical complexity, which render isolation procedures inefficient and impair large scale chemical synthesis, respectively. In the last decade, the utilization of microorganisms for the efficient and sustainable production of specific plant metabolites emerged as a promising alternative (Marienhagen and Bott, 2013; Halls and Yu, 2008; Ververidis et al., 2007).

Escherichia coli has been shown in many studies to be highly suitable as a host for the production of naringenin, a key intermediate in the flavonoids pathway (Leonard et al., 2007; Santos et al., 2011; Hwang et al., 2003; Kaneko et al., 2003). However, identifying the optimal set of heterologous genes, the best way to assemble them, and defining the best conditions for the production of flavonoids, such as naringenin and quercetin, can be laborious and highly time consuming. In order to faster find the right heterologous genes and to secure a high flavonoid production, novel high throughput screening approaches for flavonoid production are warranted.

Quantification of intracellular metabolites in the production host is often a challenge as the production of small molecules is seldom associated with a specific and measurable phenotype. In nature, various transcriptional and translational regulatory mechanisms have evolved to serve as molecular reporters in the presence of a specific ligand. Application of transcriptional regulators or RNA molecules as biosensors to correlate the detection of small molecules to the read out of a reporter protein is an upcoming method (van Sint Fiet et al., 2006; Gredell et al., 2012). These biosensors make use of the natural regulatory repertoire of the cells, as was shown for amino acids (Binder et al., 2012; Mustafi et al., 2012) or of existing promoter systems altered in their effector specificity, as in the case of mevalonate and triacetic acid lactone (Tang and Cirino, 2011; Tang et al., 2013). One of the major advantages of using fluorescent reporter proteins is the ability to use fluorescence-activated cell sorting (FACS) for high throughput screening, and thus drastically shorten the time for analyzing millions of mutant cells (Binder et al., 2012, 2013). The number of homologous transcriptional regulators is limited in *E. coli* and modifying the substrate specificity of such regulators by evolution can be time consuming. However, new biosensors can be generated using heterologous regulatory mechanisms. An example

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of transcriptional regulators that recognize plant metabolites can be found in plant symbiotic bacteria to activate nodulation gene expression necessary for nitrogen fixation (Rossen et al., 1985). Another set of transcriptional regulators in bacteria respond to plant secondary metabolites by activation of several degradation pathways for the usage as carbon sources or as a detoxification mechanism (Rao and Cooper, 1994).

This study aimed at developing biosensors for key intermediates of the flavonoid pathway. We identified two key metabolites: (i) naringenin, the major branch point common intermediate for various flavonoid derivatives (Marienhagen and Bott, 2013) and (ii) quercetin, a flavonoid of commercial interest because of its antioxidant and anti-inflammatory properties (Kumar et al., 2003; Moreira et al., 2004). Recently the transcriptional regulator FdeR from *Herbaspirillum seropedicae* SmR1 was reported to activate a naringenin degradation pathway (Marin et al., 2013). We took advantage of this transcriptional regulator to generate a biosensor for naringenin detection. Another biosensor was constructed from a transcriptional regulator which detects flavonoids, like quercetin, in *Bacillus subtilis* (Hirooka and Fujita, 2011; Hirooka et al., 2007). It regulates the transcription of *qdoI*, whose gene product is responsible for quercetin degradation (Hirooka and Fujita, 2011). Here the development of these two new biosensors, from design to *in vivo* detection of metabolites on a single cell level, is described. Our study contributes to the repertoire of transcription factor based biosensors in *E. coli*.

2. Materials and methods

2.1. Bacterial strains, media and growth conditions

Bacterial strains and plasmids are listed in Table 1. *E. coli* strains were transformed as previously described by Hanahan (1983) and cultivated in LB medium (Miller, 1972), 2xYT medium (16 g l^{-1} tryptone, 10 g l^{-1} yeast extract, 5 g l^{-1} sodium chloride) or in M9 minimal medium (Sambrook and Russel, 2001) containing 10 g l^{-1} glucose. Liquid cultures were routinely incubated in10 ml test tubes or 250-ml-baffled Erlenmeyer flasks overnight at 37 °C and 250 rpm. The *E. coli* cells transformed with each plasmid (Table 1) were selected by adding antibiotics (spectinomycin and kanamycin) to the medium at a final concentration of 50 μg ml^{-1} according to the drug resistance. *E. coli* DH5*α* (Hanahan, 1983) was used for cloning purposes and *E. coli* BL21 (DE3) (Invitrogen, Karlsruhe, Germany) and derivatives for gene expression and whole-cell biotransformation for sensor establishment. For stock cultures, 1 ml of overnight LB culture was gently mixed with 1 ml 30% (v/v) glycerol and stored at −80 °C. For analysis of the metabolite sensor response to different flavonoids the *E. coli* cells containing either pG-FdeR or p441-QdoR were cultivated in 1 ml 2xYT to an optical density at 600 nm (OD_{600}) of approximately 0.7. Then various flavonoids (naringenin chalcone was dissolved in ethanol and naringenin, dihydrokaempferol, kaempferol and quercetin were dissolved in dimethylsulfoxide (DMSO)) were added to a final concentration of 0.1 mM, unless otherwise stated, and the cultures were incubated at 37 °C for 20 h and analyzed regarding growth, fluorescence and population heterogeneity.

2.2. Recombinant DNA work

Standard methods including PCR, DNA restriction enzyme digestion and ligation were carried out according to standard protocols (Sambrook and Russel, 2001). The transcriptional regulator based biosensors were synthesized by GeneArt (Regensburg, Germany). For the construction of the biosensor the DNA region containing the native promoter and open reading frame of either QdoR or FdeR were used together with the native promoter regions whereas green fluorescent protein (GFP) or cyan fluorescent protein (CFP) were used instead of the native target gene. The *qdoR* synthetic gene was codon optimized for *E. coli*, while the nucleotide sequence of *fdeR* synthetic gene was identical to the native. The QdoR-GFP construct was sub-cloned into pSEVA441 using EcoRI and KpnI restriction sites to originate the p441-QdoR sensor plasmid. The FdeR-CFP construct was used in the original GeneArt plasmid containing kanamycin resistance.

Table 1
Strains and plasmids used in this work.

Strains, plasmids or oligonucleotides	Relevant characteristics/sequence(5′–3′)	Source or reference
Strains		
BL21(DE3)	F⁻ *ompT* $hsdS_B(r_B^-, m_B^-)$ *gal dcm rne*131 (DE3)	Invitrogen
BL-QdoR	BL21 (DE3) with p441-QdoR	This study
BL-FdeR	BL21 (DE3) with pG-FdeR	This study
BL-QdoR-FdeR	BL21 (DE3) with p441-QdoR and pG-FdeR	This study
BL-QdoR-FLS1	BL21 (DE3) with p441-QdoR and pRSF-FLS1	This study
BL-QdoR-RSF	BL21 (DE3) with p441-QdoR and pRSF-duet	This study
Plasmids		
pSEVA441	Spc^R, mcs, default, pRO1600/ColE1 origin	Silva-Rocha et al. (2013)
p441-QdoR	pSEVA441 derivative containing QdoR-GFP regulator, promoter construct	This study
pG-FdeR	Kan^R, pMK-RQ, derived from GeneArt	This study; GeneArt
pRSF-duet	Kan^R, Double T7 promoters; RSF ori	Novagen
pRSF-FLS1	pRSF-duet derivative containing the flavonol synthase gene (*fls1*) from *Arabidopsis thaliana*	Malla et al. in press
Oligonucleotides		
H132X_for	GGGTCGATNNKCTCTTCCATCG	
H132X_rev	GGAAGAGMNNATCGACCCAAGC	
F134X_for	GATCATCTCNNKCATCGAATCTGG	
F134X_rev	GATTCGATGMNNGAGATGATCGAC	
M201X_for	GCGGAGTATATGNNKAAGATTAACTATTATCCGCCG	
M201X_rev	GGCGGATAATAGTTAATCTTMNNCATATACTCCGC	
E295X_for	GGTTTTCTTGNNKCCTCCCCGTG	
E295X_rev	CGGGGAGGMNNCAAGAAAACCGG	

Oligonucleotides were synthesized by Integrated DNA Technologies, Inc (Leuven, Belgium) and are listed in Table 1. For saturation individual mutagenesis of codons His132, Phe134, Met201 and Glu295 within the intact *fls1* gene, the oligonucleotide pairs His132X_for/His132X_rev, Phe134X_for/Phe134_revX, Met201X_for/Met201X_rev and Glu295X_for/Glu295X_rev were used according to the Stratagene site-directed mutagenesis kit with the following modifications. DpnI (Fermentas) and phusion polymerase (New England Biolabs) were used.

2.3. Determination of fluorescence

The fluorescence and OD_{600} were determined in a Synergy Mx plate reader (Biotek, United States). GFP fluorescence was measured with excitation at 485 nm and emission at 515 nm with a gain set to 80. CFP emission was measured at 475 nm with an excitation wavelength of 433 nm and a gain of 70. The specific fluorescence was defined as the fluorescence per OD_{600} value (given in a.u.).

2.4. Flow cytometry

Flow cytometric measurements were performed on a FACS Aria (Becton Dickinson, SanJose, USA) with 488 nm excitation from a blue solid-state laser. Forward-scatter characteristics and side-scatter characteristics were detected as small-and large-angle scatters of the 488 nm laser, respectively. GFP and CFP fluorescence was detected using a 488-nm long-pass and a 530/30-nm band-pass filter set or a 445-nm long-pass and a 510/80-nm band-pass filter set, respectively. Data were analyzed using BD DIVA 7.0 software. The sheath fluid was sterile filtered phosphate buffered saline.

2.5. Whole-cell biotransformation and flavonoid extraction

Flaovonoid production was conducted as described previously with slight modifications (Malla et al., in press). *E. coli* strains harboring the sensor plasmid p441-QdoR and either pRSF-duet vector (BL-QdoR-RSF) or pRSF-FLS1 expression plasmid (BL-QdoR-FLS1) were pre-cultivated in 3 ml of 2xYT liquid medium with the appropriate antibiotics and incubated at 37 °C and 250 rpm overnight. The culture was then transferred to 4 ml fresh 2xYT medium at 30 °C and grown until OD_{600} of approximately 0.6. At this stage, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.5 mM to induce FLS1 expression, and the culture incubated at 30 °C for 5 h. These cells were harvested and re-suspended in M9 minimal medium supplemented with 0.5 mM IPTG in the presence of 0.25 mM dihydrokaempferol. After cultivation for 24 h at 30 °C the culture was divided and the cells were either analyzed by FACS or quenched and metabolites extracted with 1 equiv. volume of methanol for 30 min at room temperature. The total extract was centrifuged for 5 min at 16,000 × *g* and the supernatant was sterile filtered and analyzed by high performance liquid chromatography (HPLC).

2.6. HPLC analysis

The production of kaempferol in *E. coli* recombinant strains was analyzed and quantified using a high-performance liquid chromatography equipped with a Discovery® HS F5-5 column (4.6 mm by 150 mm; 5.0-μm particle size; Sigma–Aldrich) connected to a UV detector (290, 360 nm). A flow rate of 1 ml/min was used with a linear gradient of 10 mM ammonium formate pH 3.0 buffer (phase A) and acetonitrile (phase B) by the following method: 0–2 min (5% B), 2–10 min (5 to 50% B), 10–13 min (50% B), 13–16 min (50–5% B), and 16–20 min (5% B). Under these conditions, the dihydrokaempferol and kaempferol were detected at 10.7 min and 12.5 min of retention time, respectively. A calibration curve of pure kaempferol was used for quantification.

2.7. Analyzing biosensor response to altered kaempferol production

For screening of altered FLS1 activity, *E. coli* BL21(DE3) was transformed with p441-QdoR and the pRSF-FLS derivatives subjected to site-directed mutagenesis and plated on LB agar plates containing 50 μg ml^{-1} kanamycin and 50 μg ml^{-1} spectinomycin. Single colonies were inoculated into 200 μl of 2xYT medium in a 96-well plate and grown overnight at 30 °C and 350 rpm. For the main culture 5 μl of the preculture was inoculated into 145 μl 2xYT medium in a 96-well plate. After 3 h of cultivation at 30 °C *fls1* expression was induced by the addition of IPTG to a final concentration of 0.5 mM. The cells were cultivated another 4 h at 30 °C, and subsequently biotransformation was started by adding 200 μM of dihydrokaempferol. The OD_{600}, specific fluorescence and kaempferol production were measured after 16 h.

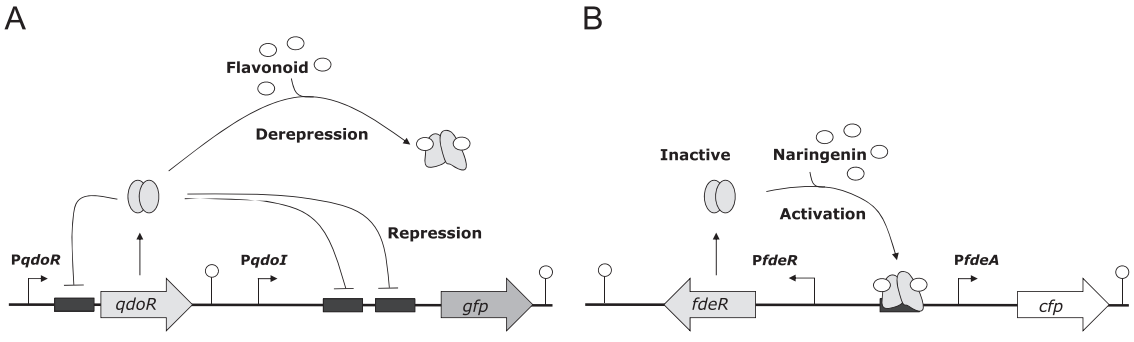

Fig. 1. Schematic views of QdoR or FdeR regulatory systems (adapted from Hirooka and Fujita, 2011). The genes, promoters, and hairpin structures probably functioning as transcriptional terminators are indicated by thick arrows, bent arrows, and stem loops, respectively. (A) Organization of the biosensor construct based on the *qdoR-qdoI* gene region. The DNA fragment covering the *qdoR* promoter, a codon optimized *qdoR* gene and the *qdoI* promoter was fused to the *gfp* gene. QdoR proteins form dimers (two ovals), and bind to QdoR boxes (dark gray boxes) located in the promoter regions repressing *qdoR* and *gfp* expression. The binding of QdoR to the QdoR boxes is inhibited by certain flavonoids (small circles), which leads to derepression of the regulon members. (B) Gene organization and proposed mechanism for the naringenin biosensor based on the *fdeR-fdeA* gene region. The DNA fragment contains the *fdeR* promoter together with the native *fdeR* gene and the *fdeA* promoter fused to the *cfp* gene. FdeR proteins presumably form dimers (two ovals), and are inactive in the absence of the effector. In the presence of naringenin (small circles) FdeR probably binds to a box located upstream of the *fdeA* promoter and activates *cfp* expression.

3. Results

3.1. Biosensor design and construction

A literature survey was conducted for the identification of potential transcriptional regulators to be used as biosensors for plant secondary metabolites. Our main assumption is that transcriptional regulators responding to plant secondary metabolites would be present in bacteria that are directly exposed to these compounds in nature, such as soil bacteria and plant symbionts. In order to compare the performance of different types of transcriptional regulators in a heterologous host, two interesting candidates were chosen based on: (i) their ability to regulate gene expression in the presence of specific effectors (flavonoids) in the native organism and (ii) their different mechanisms for regulating gene expression as either an activator or a repressor.

QdoR from *B. subtilis* acts as a repressor that can be inactivated upon binding to its effector. It regulates, together with its paralogous transcriptional regulator LmrA, the expression of the *lmrAB* operon, the *qdoR* itself and the *qdoI-yxaH* operon. QdoI was shown to have quercetin 2,3-dioxygenase activity, which is the first step in a potential quercetin degradation pathway for detoxification (Hirooka and Fujita, 2011; Hirooka et al., 2007). We have taken advantage of this regulatory system by setting the expression of the green fluorescent protein (GFP) under the control of QdoR by placing the *qdoI* promoter upstream of *gfp* (Fig. 1A). Hereby, we constructed a biosensor for quercetin and other flavonols.

FdeR from *H. seropedicae* belongs to the LysR family of transcriptional regulators and acts most likely as an activator in the presence of its effector molecule naringenin. In *H. seropedicae* FdeR activates the transcription of the *fdeABCDEFGHIJ* gene cluster, wherein *fdeA* encodes for a protein necessary for naringenin degradation (Marin et al., 2013). Construction of a naringenin biosensor was carried out by replacing *fdeA* by *cfp* encoding a cyan fluorescent protein (CFP) (Fig. 1B).

3.2. Effector range and response of the biosensors in vivo

To investigate the effector range of the constructed biosensors, *E. coli* cells harboring the respective sensor plasmid (pG-FdeR or p441-QdoR) were grown overnight in the presence of 0.1 mM of various flavonoids (naringenin chalcone, naringenin, dihydrokaempferol, kaempferol and quercetin). The specific fluorescence of the cells was measured *in vivo*, 24 h after the addition of the flavonoid (Fig. 2A). The two sensor plasmids, pG-FdeR and p441-QdoR, were active in *E. coli* and showed high expression of the fluorescent proteins CFP and GFP after addition of their respective effectors. *E. coli* cells containing the sensor plasmid pG-FdeR showed a high increase in specific fluorescence in the presence of naringenin and naringenin chalcone. These two molecules are the metabolic intermediates of the initial steps in the biosynthesis of kaempferol and quercetin (Fig. 2B). The latter molecules are recognized by QdoR resulting in *gfp* expression and therefore high specific fluorescence. For the useful application of biosensors in pathway optimization and strain screening it is essential to determine the biosensor detection range and to verify that the concentration of the effector significantly correlates with the fluorescence emitted by the reporter. These two aspects were investigated by measuring fluorescence in living cells exposed to a range of effector (Fig. 3). The minimal concentration of naringenin required to activate FdeR was 0.005 mM; moreover, a tight correlation was observed between fluorescence intensity and naringenin concentration of up to 0.3 mM (Fig. 3A). Kaempferol and quercetin activated QdoR to the same extent with a fluorescence maximum after addition of 0.1 mM. The minimal concentration of kaempferol required to activate QdoR was 0.005 mM while quercetin was detected after addition of 0.01 mM (Fig. 3B).

3.3. Measurements at the single cell level

To investigate the heterogeneity of the cell population and the potential to use this technology to screen at the single cell level, *E. coli* cultures carrying the biosensor plasmids were analyzed by FACS after 24 h of cultivation in the presence of 0.1 mM of the specified flavonoids. Cells containing the naringenin biosensor pG-FdeR (BL-FdeR) showed low fluorescent cells after incubation with kaempferol, while addition of naringenin resulted in a shift of the fluorescence of the cells towards higher blue fluorescence (Fig. 4A). The opposite behavior was observed when using cells harboring the plasmid p441-QdoR (BL-QdoR). These cells showed low green fluorescence after addition of naringenin in a well-defined histogram and a high number of fluorescent cells after addition of kaempferol (Fig. 4B). This result demonstrates the potential of using the biosensors for screening at the single cell level, because cells carrying either pG-FdeR or p411-QdoR incubated in the presence of kaempferol could clearly be distinguished from cells grown in the presence of naringenin.

3.4. Biosensor response to the in vivo production of kaempferol

The aim of a biosensor is to enable high throughput screening of strains producing a compound of interest. Thus, we set out to test

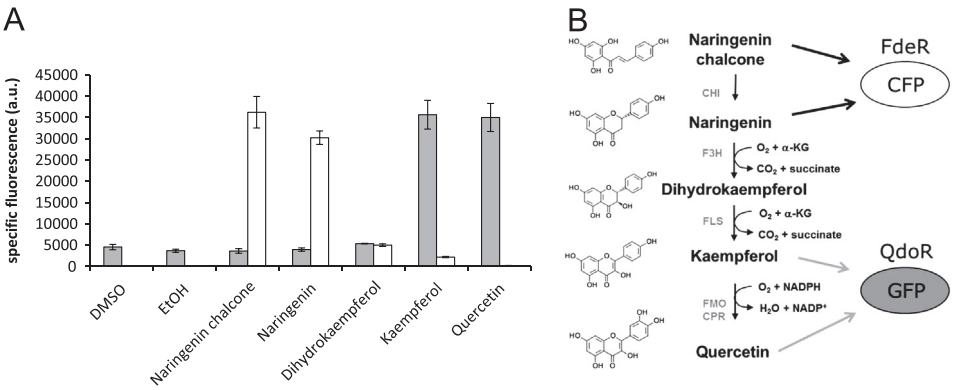

Fig. 2. (A) Effector spectrum of the constructed biosensors. The specific fluorescence of *E. coli* cells harboring a biosensor plasmid after cultivation in the presence of 0.1 mM of various flavonoids. DMSO and EtOH were used as controls since flavonoids were dissolved in either one or the other solvent. BL-QdoR (gray bars), BL-FdeR (white bars). Mean values and standard deviations from three independent experiments are shown. (B) Schematic representation of the flavonoid synthetic pathway from naringenin chalcone to quercetin; the biosensors constructed in this work and their cognate metabolites are highlighted. Abbreviations: DMSO, dimethylsulfoxide; EtOH, ethanol, α-KG, α-ketoglutarate; CHI, chalcone isomerase; F3H, flavanone 3-hydroxylase; FLS, flavonol synthase; FMO, Flavonoid 3′-monooxygenase; CPR, NADPH-cytochrome P450 reductase.

biosensor applicability for detection of flavonoid formation in designed *E. coli* strains. For this purpose the QdoR sensor plasmid was introduced in *E. coli* cells expressing a flavonol synthase (FLS1) from *Arabidopsis thaliana* (Prescott et al., 2002). FLS1 catalyzes the conversion of dihydrokaempferol to kaempferol. While QdoR responds to kaempferol but not to dihydrokaempferol (Fig. 2), its production was expected to be monitored *in vivo* using the biosensor. The *E. coli* cells containing the p441-QdoR plasmid together with either the pRSF-FLS1 plasmid (BL-QdoR-FLS1) or with the pRSF-duet vector (BL-QdoR-RSF) were analyzed at the single cell level by FACS, at 24 h after the addition of dihydrokaempferol. In BL-QdoR-FLS1 the addition of dihydrokaempferol resulted in a high number of fluorescent cells and a nearly symmetric distribution of the fluorescence signal whereas the negative control BL-QdoR-RSF showed lower fluorescence, indicating that sensor activity strictly requires a functional FLS1 (Fig. 5). To validate our biosensor results, production of kaempferol from cell extracts of cells grown in the presence of 0.25 mM dihydrokaempferol was analyzed by HPLC. Cultures of *E. coli* strain BL-QdoR-FLS1 were confirmed to produce 0.1 mM kaempferol after 24 h, while the latter metabolite was not detected in extracts of BL-QdoR-RSF. These results confirm the output of the QdoR based biosensor for kaempferol *in vivo* detection.

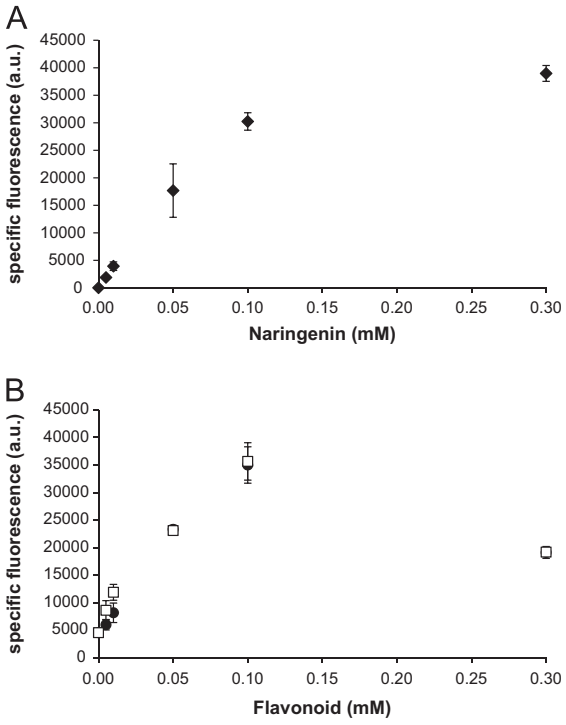

Fig. 3. Fluorescence intensity as a function of effector concentration. The specific fluorescence was measured after 18 h of cultivation in the presence of selected flavonoids (A) The BL-FdeR cells were analyzed regarding their response to naringenin. (B) The BL-QdoR cells were analyzed regarding their response to kaempferol (white squares) or quercetin (black dots). Mean values and standard deviations from three independent experiments are shown.

3.5. Analyzing biosensor response to altered kaempferol production

To validate the applicability of the biosensors for high throughput screening a correlation between the ability of the cells to produced kaempferol and the biosensor output was established. The fluorescence signal in response to a range of produced kaempferol concentrations was evaluated using a FLS1 mutant library generated by site directed mutagenesis of amino acids involved in substrate binding (Chua et al., 2008). This library was transformed together with the sensor plasmid p441-QdoR into BL21(DE3) and the resulting strains

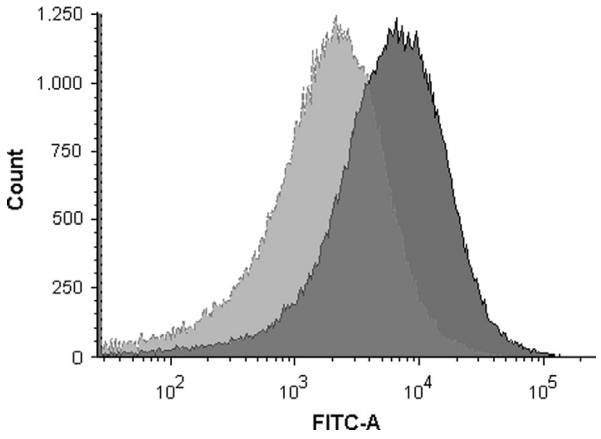

Fig. 5. FACS-generated histogram showing GFP fluorescence. The cells of BL-QdoR-FLS1 (dark gray) and BL-QdoR-RSF (light gray) were examined. The fluorescence was measured 24 h after the addition of 0.25 mM dihydrokaempferol. Representative histogram of three independent experiments is shown.

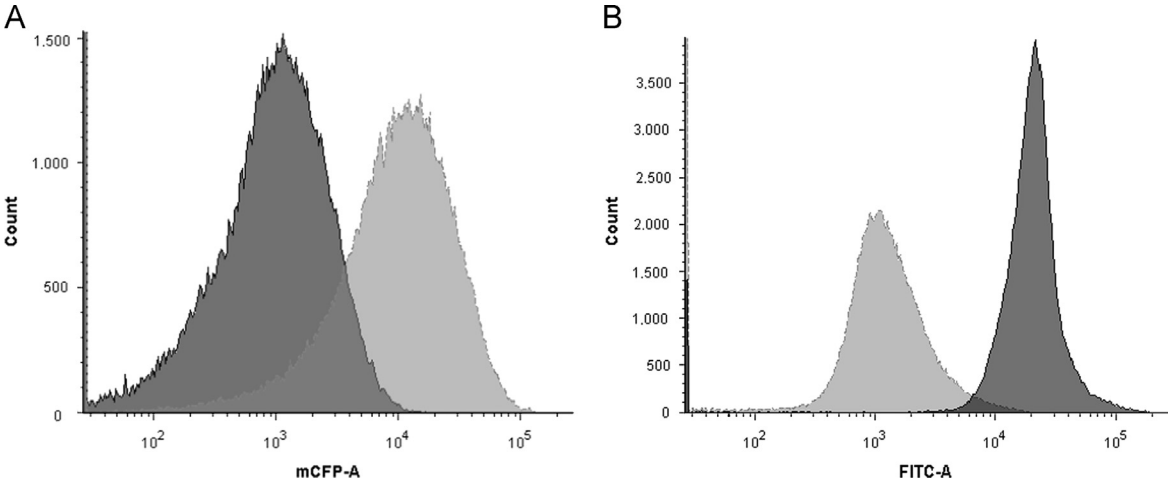

Fig. 4. FACS-generated histograms displaying the fluorescence signals. The cells of BL-FdeR and BL-QdoR were incubated 24 h with 0.1 mM of the respective flavonoid and analyzed by flow cytometry. (A) Blue fluorescence of BL-FdeR after incubation with naringenin (light gray) or kaempferol (dark gray). (B) Green fluorescence of BL-QdoR after addition of naringenin (light gray) or kaempferol (dark gray). Representative histograms of three independent experiments are shown.

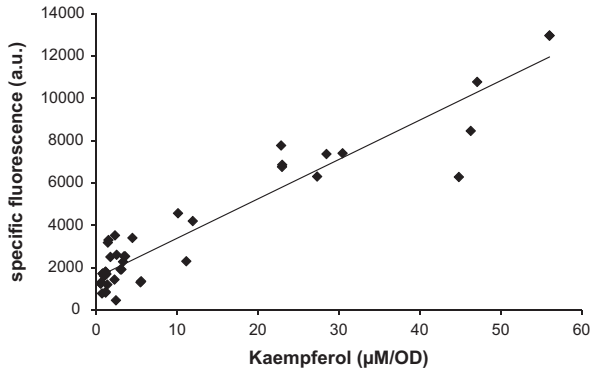

Fig. 6. Correlation between concentration of kaempferol produced and specific fluorescence measured 16 h after the addition of 200 μM dihydrokaempferol in *E. coli* cells containing plasmids p441-QdoR and a mutant library of pRSF-FLS.

were analyzed regarding their specific fluorescence in 96-well plates and kaempferol amounts after 16 h of biotransformation. Under these experimental settings, a tight correlation between the specific fluorescence emitted and the concentration of kaempferol produced by the cells was observed. These results show that the specific fluorescence is dependent on the activity of the FLS1 enzyme and the kaempferol produced (Fig. 6). The highest specific fluorescence with 12,967 arbitrary units and the highest product formation with 56 μM of kaempferol produced per OD_{600} were measured for cells harboring the wild type FLS1 enzyme.

4. Discussion

In this study, we showed the potential of using heterologous transcriptional regulators to design biosensors for the detection of plant secondary metabolites. We have constructed two biosensors: one for the key flavonoid pathway intermediate naringenin and another one for kaempferol and quercetin.

QdoR belongs to the TetR family of transcriptional regulators, whereas FdeR belongs to the LysR family. In previous studies, three biosensors were evolved from LysR type regulators, but also two of different types, the ROK (Binder et al., 2012) and the Lrp type (Mustafi et al., 2012). This diversity shows the ability to use different transcriptional regulators as biosensors. More than 230 transcriptional regulators have been reported in *E. coli*, many of them detecting small molecules (Binder et al., 2012). The availability of endogenous transcriptional regulators is limited compared to a vast number of chemical structures that can potentially be produced in *E. coli*. In nature, transcriptional regulators evolved to recognize enormous numbers of substrates, including aromatic compounds, like catechol or 2-nitrotoluene (Tropel and van der Meer, 2004) or flavonoids secreted by legumes (Kape et al., 1992; Rossen et al., 1985; Zaat et al., 1987). Tapping nature's transcriptional regulators potential to design new biosensors is expected to accelerate screening procedures and thereby considerably shorten strain development for the production of high-value compounds.

Screening and analysis of bacterial cells tailored for metabolite production using a biosensor requires that the relationship between the effector molecule input and reporter output is well understood. The transcriptional regulator FdeR was shown to induce *fdeA* transcription after addition of 0.2 mM naringenin in *H. seropedicae* SmR1, but the minimal concentration for activation was not investigated (Marin et al., 2013). In *E. coli*, the linear detection range was between 0.005 and 0.1 mM of naringenin and saturation occurred between 0.1 and 0.3 mM. Efforts to engineer the production of the plant flavonoid naringenin in *E. coli* has previously been reported, while the first generation strain produced 0.3 mM of naringenin, an improved mutant had a 5.5-fold higher titer (Xu et al., 2011). These concentrations are above the saturation limit of the FdeR biosensor, impairing its utilization to further improve naringenin titers and yields. However, naringenin is a key intermediate in the synthesis of numerous flavonoids at high demand for the food and pharmaceutical industry (Bland, 1996). Importantly, the naringenin biosensor can be used to screen novel enzymes for activity or for optimization of production of molecules downstream in the biosynthesis pathway, where the accumulation of the intermediate, naringenin, can be monitored.

The *in vivo* assays of QdoR in *E. coli* showed a detection limit of 0.005 and 0.01 mM of kaempferol or quercetin, respectively. Our results were comparable to those reported using an *in vitro* assay, in which the repressor was found to have a K_d of 1nM for the intergenic region of *qdoI* and a inhibition constant (K_i) of 0.47 mM quercetin. However, some dissociation of the protein DNA complex was detected after addition of 0.06 mM quercetin *in vitro* (Hirooka et al., 2007). This concentration range makes the system suitable for the analysis and screening of strains engineered for the production of quercetin and/or kaempferol, such as those described (Malla et al., in press; Leonard et al., 2006). The specific fluorescence showed an optimum after addition of 0.1 mM of the effector, while addition of a higher flavonoid concentration led to a decrease in specific fluorescence. This is probably due to the low water solubility of kaempferol and quercetin.

Notably, the biosensor technology enables the use of fluorescence activated cell sorting to identify cells producing an expected compound in a high throughput manner. We showed that cell populations incubated with inducing and non-inducing flavonoids could be distinguished from each other. Hence, we were able to, for the first time, use biosensors to detect fluorescence coupled to flavonoid production *in vivo*. An *E. coli* strain, BL-QdoR-FLS1, expressing a plant-derived flavonol synthase (FLS1) and the QdoR-sensor, produced 0.1 mM of kaempferol after 24 h of cultivation. The cells carrying FLS1 showed high fluorescence levels in contrast to cells harboring pRSF-duet vector.

Furthermore, the QdoR sensor was successfully utilized for characterizing a small library of FLS1 mutants with altered kaempferol production in 96-well plates *in vivo*. The significant correlation observed between the fluorescence of the cells and the kaempferol they produced clearly indicate that this technology can be applied for *in vivo* high throughput screening. The power of *in vivo* selection of enzymes for metabolic rerouting has been evidenced in a number of recent reports (Tang et al., 2013; Binder et al., 2012, 2013) and strengthened by the observations that *in vitro* kinetics and *in vivo* product formation do not always correlate (Schendzielorz et al., in press).

Overall, our results show that the flavonoid biosensor based on the transcriptional regulator QdoR can be used to isolate gene products and optimize pathways involved in flavonoid synthesis. Furthermore, coupling of these novel biosensors to FACS can be exploited to screen larger libraries of up to 10^9 cells. This enabling technology is expected to facilitate studies aiming at identifying novel enzymes catalyzing the formation of kaempferol, or at optimizing the production of naringenin and quercetin and thereby shorten the time to develop next generation cell factories for flavonoid production.

5. Conclusion

The ability to use heterologous transcriptional regulators for the detection of small molecules, and in particular of the biosensors herein designed will expedite and facilitate the development of novel strain as efficient cell factories for production of flavonoids.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ymben.2013.10.011.

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