APTAMERS AND RIBOSWITCHES OF LUTEOLIN AND OTHER NARINGENIN DERIVED FLAVONOIDS FOR IN VITRO AND IN VIVO SENSING

FIELD OF THE INVENTION

[0001] The present invention generally relates to an aptamer and a riboswitch comprising the aptamer. In particular, the present invention relates to an aptamer and a riboswitch comprising the aptamer for detecting luteolin and other naringenin derived flavonoids.

BACKGROUND

[0002] There is an increasing demand for sustainable and environmentally friendly solutions to produce essential chemicals, leading to the use of microbes/enzymes that have been engineered to produce high-value chemicals from renewable feedstocks through synthetic biology/metabolic engineering. The global synthetic biology market is forecasted to grow from US$5.3 billion in 2019 to US$18.9 billion by 2024 (BCC Research). Flavonoids are a family of secondary metabolites found naturally in plants and fungi [1]. Among them, luteolin is gaining attention due to its numerous health benefits, including antioxidant, anti-inflammatory, anticancer, neuroprotective, analgesic, and cardiovascular protective effects [2–4]. However, traditional methods of producing flavonoids, including luteolin, rely heavily on plant extraction, which is inefficient and unsustainable [5]. As a result, there is a growing interest in microbial production, sustainable and environmentally friendly solutions that are characterized by rapid growth rates and cost-effective scalability [6–8]. Although earlier studies have engineered microbes/enzymes to produce flavonoids from renewable feedstocks using synthetic biology and metabolic engineering approaches [5,9–11], further improvement is required to achieve industrial-scale production. Specifically, microbial production of valuable flavonoid luteolin remains underexplored, with low titer highlighting the need for further strain and enzyme engineering [12,13].

[0003] Despite the recent advancement in synthetic biology and computational tools, predictively designing microbial strains remains challenging due to the complexity of biological systems. Consequently, large-scale screening is often necessary to identify high-performance strains. Analytical techniques such as high-performance liquid

chromatography (HPLC) are commonly used to quantify flavonoids. While these methods are precise and accurate, they suffer from timeconsuming, resource intensive, and complex procedures, significantly limiting their application in high throughput screening.

[0004] Different methods have been used to screen for the desired strains, including traditional manual screening, with the assistance of automated colony picker, and modern cell analysis technique fluorescent-activated cell sorting (FACS). Conventional method for screening largely relies on manual work and using precise but low throughput titration equipment such as High-performance liquid chromatography (HPLC) and Liquid Chromatography Mass Spectrometry (LCMS). While HPLC or LCMS instruments are sensitive, they are bulky, expensive, and difficult to use, as well as requiring regular maintenance. More importantly, they are low throughput, as each sample requires around 30 minutes to process, in addition to tedious sample preparation steps. The process for HPLC or LCMS is laborious, costly, and, more importantly, very inefficient (~30 strains per week). Electrochemical sensors for the detection of flavonoids such as luteolin based on the changes in electrical properties of electrode have been recently developed for food quality testing application. While electrochemical sensors would allow easier detection of the molecules, they are limited to *in vitro* (i.e. outside live cells) detection and can be difficult to fabricate. More advanced approaches, such as microfluidic systems or flow cytometry can enable ultra-high throughput screening (millions of cells). The comparison of different sensing technologies is shown in Table 1.

[0005] **Table 1: A comparison of the different sensing/detection methods**

Features	**Common practice (HPLC/LCMS)**	**Raman (each chemical has unique Raman spectrum)**	**Electrochemical based biosensor**	**DNA/RNA based biosensor**
Sensing device cost	Expensive	Expensive	Medium	Extremely low
Operation procedure	Tedious	Normal	Normal	Easy
Sensing time	20-30 mins	A few seconds through to several days	A few seconds to several minutes	A few milliseconds
Device footprint	Large	Large	Medium	Extremely small
Single cell sensing	No	No	No	Yes
***In vivo* sensing**	No	No	No	Yes
Sensitivity	Very high	Medium	Medium	High

[0006] To overcome these limitations, genetically encoded biosensors have been developed. These biosensors enable high throughput screening of engineered strains, real-time monitoring of product concentration, and regulation of the metabolic pathways to enhance production performance [14–18]. Riboswitches offer several advantages over protein-based transcriptional factor biosensors [19,20], including lower metabolic burden [21,22], faster responses time, and reduced off-target effects [23]. Although riboswitches usually exhibit relatively lower sensitivity compared to aptamer-based biosensor [24,25] (also refer to Table 2), they offer distinct advantages, such as the reporter fluorescence proteins (e.g., GFP) expressed by riboswitch are more stable than fluorogenic RNA in bacterial cells [26]. Besides, riboswitches eliminate the need for external dyes. In addition, aptamer-based sensors are not suitable for regulating gene expression, whereas riboswitches can modulate gene expression in response to specific ligand binding. A typical riboswitch consist two components: an aptamer domain allows binding of target molecule and an gene regulatory expression domain generates readout or regulates the downstream genes [27]. Ligand binding triggers structural rearrangements in the RNA, altering accessibility to regulatory elements and enabling control over translation [28,29]. Since riboswitches can be used in the bacteria cells to measure intracellular metabolite concentrations, they have been used to screen for highly productive strains or enzymes [30–33]. Synthetic riboswitches have been developed for a variety of ligands [34–52]. However, in the flavonoid family, synthetic riboswitches have only been developed for naringenin [51]. Currently, no riboswitch has been developed for detecting or regulating gene expressions in response to other flavonoids, such as luteolin.

[0007] **Table 2. Sensitivity comparison of different types of biosensors.**

Sensor Type	Sensitivity Range	Mechanism	Key Applications	Reference
Riboswitch	μM to nM	RNA conformational change	Intracellular sensing and gene regulation	[27, 28]
Transcription Factor Sensor	μM to nM	Protein-DNA interaction	Intracellular sensing and gene regulation	[19, 20]
Aptamer-Based Sensor	pM to nM	Synthetic RNA/DNA binding	Diagnostics, environmental monitoring	[24, 25]

[0008] To fill this gap, synthetic riboswitches that sensitively and specifically respond to luteolin have been developed. The present invention discloses novel RNA-based biosensors (synthetic riboswitches) for the detection of flavonoids such as luteolin. The luteolin-binding aptamers were first found using systematic evolution of ligands by exponential enrichment (SELEX) [53]. These aptamers were integrated into a green fluorescence protein (GFP) expression module to construct the riboswitches. Functional riboswitches were selected using replica plating approach [54]. This method involves comparing the fluorescence of the colonies in the absence and presence of luteolin. Two functional riboswitches were successfully developed, exhibiting operational range of 1.75–105 μM concentration of luteolin, with a maximum fold activation of 9.6. In addition, the specificity of the riboswitches to luteolin was evaluated to ascertain their utility for monitoring flavonoid biosynthesis pathways. To assess their stability and reproducibility, the biosensors were tested under varying conditions, including different host strains, temperatures, and culture medium. The results showed their robust performance and reliability. Moreover, the sensing of intracellular luteolin produced by engineered E. coli was demonstrated. The biosensor output achieved a good correlation (R^2 = 0.93) with luteolin concentrations measured by HPLC. To demonstrate the versatility of the biosensors, the intracellular application was extended to apigenin, where the biosensor effectively distinguished intracellular apigenin concentrations produced by E. coli. This is the first work that developed functional synthetic aptamers and riboswitches for luteolin. These tools enable precise detection of luteolin and regulation of gene expression, offering potential for screening and engineering flavonoid-producing strains and enzymes. Overall, the aptamers and riboswitches developed here represent a valuable toolkit for flavonoids research and applications.

SUMMARY

[0009] To fill the gap that no RNA-based biosensor for the detection of luteolin and other naringenin derived flavonoids is available, novel synthetic riboswitches that respond to luteolin and other naringenin derived flavonoids, as well as the aptamers that specifically bind to luteolin and other naringenin derived flavonoids, have been developed. Novel RNA sequences that bind with luteolin and other naringenin derived flavonoids and function as riboswitches were determined. The responses of the riboswitches to different luteolin concentrations have been characterized and critical

parameters, such as sensitivity, specificity, dynamic range and detection limit have been determined (see results section). This is the first time that functional luteolin riboswitches for *in vivo* sensing are developed. The ability to regulate gene expression and detect luteolin and other naringenin derived flavonoids *in vivo* will be extremely useful in the screening and engineering of luteolin-producing strains/enzymes. Furthermore, the luteolin aptamers and riboswitches disclosed herein have the versatility to be used for other applications, such as *in vitro* luteolin and other naringenin derived flavonoids detection. Overall, the disclosed aptamers and riboswitches solve an important problem in the production and detection of luteolin and other naringenin derived flavonoids. Very importantly, the riboswitches also enable ultra-high throughput screening. As such, the disclosed aptamers and riboswitches of luteolin and other naringenin derived flavonoids would have significant impact in both laboratory and industrial settings.

[0010] In one aspect, the present disclosure refers to an aptamer capable of binding to luteolin and other naringenin derived flavonoids, wherein the aptamer is: **an RNA aptamer** having a nucleotide sequence selected from the group consisting of: GUCCNNNNUCAGUCNNNNGACUGAGGAU (SEQ ID NO: 1), and AUGCUGGNNNNNCUAGUAUNNNNACAUNNNNAUGU (SEQ ID NO: 2); or **a DNA aptamer** having a nucleotide sequence selected from the group consisting of: GTCCNNNNTCAGTCNNNNGACTGAGGAT (SEQ ID NO: 3), and ATGCTGGNNNNNCTAGTAT (SEQ ID NO: 4); wherein for SEQ ID NO: 1 and 2, N is one nucleotide selected from the group consisting of A, G, C and U, and wherein for SEQ ID NO: 3 and 4, N is one nucleotide selected from the group consisting of A, G, C and T.

[0011] In another aspect, the present disclosure refers to a riboswitch comprising the RNA aptamer as disclosed herein, a ribosomal binding site (RBS) and a reporter; wherein the RNA aptamer comprises the nucleotide sequence of SEQ ID NO: 1 or SEQ ID NO: 2.

[0012] In another aspect, the present disclosure refers to a DNA expression cassette for the regulation of the expression of the reporter as disclosed herein, comprising a promoter, a DNA aptamer as disclosed herein, a DNA sequence encoding the RBS and a DNA sequence encoding the reporter of the riboswitch as disclosed herein.

[0013] In another aspect, the present disclosure refers to a composition for detecting luteolin and other naringenin derived flavonoids, comprising the riboswitch as disclosed herein, and a translation mixture.

[0014] In another aspect, the present disclosure refers to a composition for detecting luteolin and other naringenin derived flavonoids, comprising the DNA expression cassette as disclosed herein, a transcription mixture and a translation mixture.

[0015] In another aspect, the present disclosure refers to a microbe containing the riboswitch as disclosed herein or the DNA expression cassette as disclosed herein.

[0016] In another aspect, the present disclosure refers to a method of detecting luteolin and other naringenin derived flavonoids produced in a microbe, comprising (a) introducing the riboswitch as disclosed herein or the DNA expression cassette as disclosed herein into the microbe, wherein when the microbe produces the luteolin and the other naringenin derived flavonoids in an effective amount, the luteolin and the other naringenin derived flavonoids specifically bind the aptamer to induce a conformational change in the riboswitch that allows translation of the reporter into a reporter protein; and (b) detecting the reporter protein, wherein the presence of the reporter protein is indicative of detection of the luteolin and other naringenin derived flavonoids.

[0017] In another aspect, the present disclosure refers to a method of detecting luteolin and other naringenin derived flavonoids produced by a microbe, comprising (a) incubating the microbe with the composition as disclosed herein in a medium, wherein when the microbe produces and releases the luteolin and the other naringenin derived flavonoids in an effective amount into the medium, the luteolin and the other naringenin derived flavonoids specifically bind the aptamer of the riboswitch to induce a conformational change in the riboswitch that allows translation of the reporter into a reporter protein; and (b) detecting the reporter protein, wherein the presence of the reporter protein is indicative of detection of the luteolin and other naringenin derived flavonoids.

[0018] In another aspect, the present disclosure refers to a method of detecting luteolin and other naringenin derived flavonoids produced by a first microbe, comprising (a) incubating the first microbe and the microbe as disclosed herein in a medium; wherein when the first microbe produces and releases the luteolin and the other naringenin derived flavonoids in an effective amount into the medium, the luteolin and the other naringenin derived flavonoids specifically bind the aptamer of the riboswitch produced by the

microbe as disclosed herein to induce a conformational change in the riboswitch that allows translation of the reporter into a reporter protein; and (b) detecting the reporter protein, wherein the presence of the reporter protein is indicative of detection of the luteolin and the other naringenin derived flavonoids.

[0019] The biosensor disclosed herein offers several significant advantages. In addition to being cheaper to fabricate and simpler to use, this invention can detect the luteolin both inside (*in vivo*) and outside (*in vitro*) the cells. Moreover, the biosensor is capable of high throughput screening at single cells level, which is not possible/extremely challenging with most other luteolin detection methods (HPLC, LCMS, electrochemical based biosensor). In addition, the biosensor as disclosed herein can be easily fabricated at low cost (about SGD 0.0035 per sensing reaction), and it is possible to grow the cell to expand the biosensor for larger quantity measurement. Further, the biosensor as disclosed herein has a wide dynamic sensing range of 1.75-120 μM, that is sufficient to cover the working concentration for luteolin detection and relevant strain/enzyme screening. Finally, the biosensor as disclosed herein enables ultra-high throughput screening for strain/enzymes that produce luteolin and the other naringenin derived flavonoids.

BRIEF DESCRIPTION OF THE DRAWINGS

[0020] The invention will be better understood with reference to the detailed description when considered in conjunction with the non-limiting examples and the accompanying drawings, in which:

[0021] **Figure 1** shows *In vitro* selection of luteolin aptamer. A) SELEX workflow to identify luteolin aptamer. The ssDNA oligos were incubated with luteolin conjugated beads for binding. After the unbound oligos were washed away, the bound oligos were amplified using PCR. The amplified oligos were then purified via gel electrophoresis and used for the next round of SELEX. B) Luteolin conjugation onto the beads – luteolin concentration before and after binding. After conjugation, about 90% of the luteolin was bound to the beads. C) PCR yield of SELEX at different rounds. The PCR yield increased with more rounds of SELEX, and saturated at round 13. Data information: The experimental data were represented as mean ± S.D. (n = 3). Error bars indicate the standard deviations of three technical replicates. Statistical significances of ***$P < 0.001$ were calculated based on two-sample unpaired t-test.

[0022] **Figure 2** illustrates Luteolin biosensor development. A) An illustration of luteolin biosensor's sensing mechanism. In the absence of luteolin, riboswitch forms a hairpin structure and blocks the RBS, inhibiting translation. In the presence of luteolin, it binds to the riboswitch and causing conformation change that exposes the RBS, enabling translation. B) Selected biosensors' fluorescence activation at 35 μM luteolin, measured after 16 hours of incubation. Both LB1 and LB2 showed significant GFP expression when exposing to 35 μM luteolin. Data information: The experimental data were represented as mean ± S.D. (n = 3). Error bars indicate the standard deviations of three technical replicates. Statistical significances of ***P < 0.001 were calculated based on two-sample unpaired t-test.

[0023] **Figure 3** illustrates the characterization of the luteolin biosensors. (A) Dose response of LB1 to different luteolin concentration, represented by the GFP/OD at 16 hours. (B) Dose response of LB2 to different luteolin concentration, represented by the GFP/OD at 16 hours. Overall, LB 1 exhibits greater sensitivity compared to LB2, while LB2 achieved higher fold activation than LB1. Both LB1 and BL2 demonstrated good curve fitting with Hill equation. GFP/OD = $K_{max}\frac{Lut}{Lut+EC50}$ where K_{max}, EC_{50}, and Lut refer to maximum GFP/OD, luteolin concentration to achieve half-maximum GFP/OD, and concentration of luteolin. Data information: The experimental data were represented as mean ± S.D. (n = 3). Error bars indicate the standard deviations of three technical replicates.

[0024] **Figure 4** illustrates the response of the developed biosensors to other flavonoids. (A) The fold activation of LB1 to different flavonoids at different concentrations. (B) The fold activation of LB2 to different flavonoids at different concentrations. Both LB1 and LB2 showed significantly higher fold activation towards luteolin than other flavonoids. (C) Specificity of luteolin LB1 and LB2 in the presence of luteolin, apigenin, eriodictyol, and naringenin. Both LB1 and LB2 showed significantly higher specificity towards luteolin than other flavonoids. Data information: The experimental data were represented as mean ± S.D. (n = 3). Error bars indicate the standard deviations of three technical replicates. Statistical significances of ***P < 0.001 were calculated based on two-sample unpaired t-test.

[0025] **Figure 5** illustrates luteolin biosensor for intracellular sensing. (A) Illustration of the luteolin producing genetic circuits and biosensor sensing mechanism. The *E. coli* harbouring both luteolin-producing plasmid and biosensor. FNS will be expressed when

adding IPTG and converts the eriodyctiol to luteolin. Luteolin further binds to the biosensor and triggers GFP translation. B) Sensor response when different IPTG concentration was added. Increasing IPTG concentrations leads to higher GFP expression levels. C) Luteolin produced by the *E. coli* at different IPTG induction concentrations, measured by HPLC. Increasing IPTG concentrations leads to higher luteolin production. D) The correlation between luteolin biosensor response and luteolin produced by *E. coli*. The biosensor's signal showed strong linear correlation with the luteolin concentration measured by HPLC. Data information: The experimental data were represented as mean ± S.D. (n = 3). Error bars indicate the standard deviations of three technical replicates. Statistical significances of ***P < 0.001 were calculated based on two-sample unpaired t-test.

[0026] **Figure 6** illustrates HPLC standard curve for naringenin and luteolin. A) Strong linear correlation between HPLC reading and luteolin concentration. B) Strong linear correlation between HPLC reading and apigenin concentration.

[0027] **Figure 7** illustrates the absorption spectrum of luteolin, measured by spectrophotometer. The peak absorption wavelength appeared at around 350 nm.

[0028] **Figure 8** illustrates the luteolin absorption standard curve, measured by spectrophotometer. Data information: The experimental data were represented as mean ± S.D. (n = 3). Error bars indicate the standard deviations of three technical replicates.

[0029] **Figure 9** illustrates the results of testing the stability of the luteolin-beads conjugation. After the luteolin-beads binding reaction using 140 μM, there was about 15 μM luteolin left in the supernatant. The beads were washed with PBS to remove the unbound luteolin. The supernatant of the conjugation beads was measured before starting subsequent SELEX round 4 and round 10, as well as the after 3 months storage at 4 °C. The results showed that minimal luteolin was released to the supernatant, confirming the long-term stability of the conjugation. Error bars indicate the standard deviations of three technical replicates.

[0030] **Figure 10** illustrates the auto-fluorescence of flavonoids. The auto-fluorescence of flavonoids were measured at concentration of 105 μM, which was the maximum concentration used in this study. The GFP was expressed by the E. coli at OD of 1. The excitation wavelength was set at 470 nm. The emission wavelengths of flavonoids do not overlap with GFP. Further, the fluorescence of the flavonoids was much lower as compared to GFP.

[0031] **Figure 11** illustrates the replica plate approach. Grid was drawn onto the petri dishes. The satellite colonies were scraped up from the parental plate without luteolin. The satellite colonies were transferred onto the daughter plate with 35 μM luteolin. The colonies which had increased fluorescence were selected for further characterization.

[0032] **Figure 12** illustrates the time response of the selected biosensors, characterized in liquid LB broth medium. 16 hours was sufficient to see the GFP difference in the absence and presence of luteolin.

[0033] **Figure 13** illustrates the aptamer structure of LB 1 and LB2. A) Aptamer of LB1 in DNA form. B) Aptamer of LB1 in RNA form. C) Aptamer of LB2 in DNA form. D) Aptamer of LB2 in RNA form. The aptamer secondary structures of LB1 and LB2 were visualized in DNA and RNA forms using RNAfold [61]. Both aptamers retained similar structures when converting from DNA to RNA.

[0034] **Figure 14** illustrates the fold activation of luteolin biosensors. Both LB1 and LB2 showed responsiveness at a low concentration of 1.75 μM and exhibited increasing GFP expression with raising luteolin concentrations. Data information: The experimental data were represented as mean ± S.D. (n = 3). Error bars indicate the standard deviations of three technical replicates.

[0035] **Figure 15** illustrates the time response of LB1 and LB2 to different luteolin concentrations. Both LB1 and LB2 started to show responsiveness within 50-60 min, and the difference in GFP expression levels maintained for over 16 hours.

[0036] **Figure 16** illustrates the dose-response of the biosensors in E. coli 10-beta (C3019, NEB). Both LB1 and LB2 exhibited responsiveness at a low concentration of 1.75 μM, and GFP expression increased at higher luteolin concentration, with lower fold activation than in E. coli Acella. These results demonstrated that both LB1 and LB2 in E. coli 10-beta hold their ability to detect the luteolin concentrations. Data information: The experimental data were represented as mean ± S.D. (n = 3). Error bars indicate the standard deviations of three technical replicates.

[0037] **Figure 17** illustrates the performance of LB1 and LB2 in M9 culture medium. This work primarily focused on the utility of the biosensors in LB medium, given its relevance to microbial production conditions. To further evaluate their stability and reproducibility, LB1 and LB2 were also characterized in M9 culture. Both biosensors demonstrated the capability to distinguish between different luteolin concentrations. The GFP/OD signal dropped after around 4 hours, likely due to the limited nutrition in M9.

Regardless, the differences in GFP expression levels maintained for over 30 hours, showcasing the biosensors' robust long-term performance.

[0038] **Figure 18** illustrates the biosensors specificity. A) Luteolin biosynthesis pathways related to flavonoids. B) Specificity of LB1. C) Specificity of LB2. Both LB1 and LB2 showed much higher responsiveness to luteolin as compared to other flavonoids.

[0039] **Figure 19** illustrates the the applications of biosensors on other flavonoids. A) In the flavonoid biosynthesis pathway as seen in Figure 18A, in the presence of enzyme FNS, adding naringenin as substrate will lead to the production of apigenin. B) Biosensor response when different IPTG concentration was added. C) Apigenin produced by the E. coli at different IPTG induction concentrations, measured by HPLC. D) The correlation between biosensor response and apigenin produced by E. coli. Data information: The experimental data were represented as mean ± S.D. (n = 3). Statistical significances of ***P < 0.001 and **P < 0.01 were calculated based on two-sample unpaired t-test.

[0040] **Figure 20** illustrates the establishment of correlation between biosensor fluorescence output and HPLC analysis. First, cell culture was continuously incubated and monitored using a microplate reader. At the end point, the cell culture was extract for HPLC analysis.

[0041] **Figure 21** are bar graphs illustrating the biosensor performance for detecting luteolin at (A) 18 °C, (B) 25 °C, (C) 30 °C, (D) 37 °C, and (E) 40 °C. The biosensor showed good performance at 18 °C, 25°C, 30 °C, 37 °C, but the performance declined at 40 °C.

DETAILED DESCRIPTION

[0042] There is a growing interest in engineering microbes to produce health-beneficial flavonoids such as luteolin, which has anti-oxidative, anti-tumor, and cardiac protective effects. However, it remains challenging to engineer strains or enzymes (producer) to achieve high yields, largely due to the inherent complexity of biological systems. To address this challenge, genetically encoded biosensors offer a promising solution by enabling high throughput screening and pathway optimization to enhance producer performance. However, there is a lack of sensitive and specific biosensors for the detection of luteolin. To fill this gap, this disclosure describes the development of synthetic riboswitches as genetically encoded biosensors that can activate gene expression in response to luteolin. Using SELEX and replica plating approaches, two

functional synthetic riboswitches were developed with detection range from 1.75 to 105 μM. The biosensors showed good sensitivity with half-maximal effective concentrations (EC50) of 11.71 μM and 17.62 μM, respectively. The riboswitches responded specifically to luteolin and exhibited a robust activation dynamic, achieving up to a 9.6-fold change. Further, the application of the riboswitches in detecting intracellular luteolin produced by engineered E. coli has been demonstrated, with fluorescence output showing a strong correlation ($R2 = 0.93$) with luteolin concentrations measured by HPLC. The stability and reproducibility of the biosensors were evaluated, showing their reliability under various conditions. Additionally, the application of the biosensors was extended to another flavonoid, apigenin, showcasing their versatility. Overall, these luteolin biosensors addressed a critical limitation in flavonoid. Their demonstrated properties hold promise for diverse applications in the food, pharmaceutical, and cosmetic industries, including large-scale screening of luteolin samples, detection of luteolin and other naringenin derived flavonoids for food quality control, high throughput screening for the strains with high performance, real-time pathway monitoring and regulation, and the metabolic engineering of flavonoid-producing microbes, and so on.

[0043] In one aspect, the present disclosure refers to an aptamer capable of binding to luteolin and other naringenin derived flavonoids, wherein the aptamer is: **an RNA aptamer** having a nucleotide sequence selected from the group consisting of: GUCCNNNNUCAGUCNNNNGACUGAGGAU (SEQ ID NO: 1), and AUGCUGGNNNNNCUAGUAUNNNNACAUNNNNAUGU (SEQ ID NO: 2); or **a DNA aptamer** having a nucleotide sequence selected from the group consisting of: GTCCNNNNTCAGTCNNNNGACTGAGGAT (SEQ ID NO: 3), and ATGCTGGNNNNNCTAGTAT (SEQ ID NO: 4); wherein for SEQ ID NO: 1 and 2, N is one nucleotide selected from the group consisting of A, G, C and U, and wherein for SEQ ID NO: 3 and 4, N is one nucleotide selected from the group consisting of A, G, C and T.

[0044] As used herein, the term "aptamer" refers to a nucleic acid or oligonucleotide molecule that binds to a ligand such as luteolin. Aptamers are derived from an *in vitro* evolutionary process (e.g., SELEX, (Systematic Evolution of Ligands by EXponential Enrichment), described in U.S. Patent No. 5,270,163), which selects for target-specific aptamer sequences from large combinatorial libraries. Generally, generation of aptamers by SELEX involves the following steps: (1)

Aptamer (nucleic acid, DNA/RNA) pools are incubated with target ligand (such as luteolin) for binding. (2) Target ligand (such as luteolin) are thoroughly washed to remove non-binding species. (3) Target ligand (such as luteolin)-bound aptamers are eluted from the target. (4) Amplification of candidate aptamers by PCR (DNA) or RT-PCR and transcription (RNA). Steps (1)-(4) is repeated for multiple rounds to deplete non-binders and enrich for target-binders. Following the final round of selection, binding nucleotides are cloned and sequenced to determine the specific nucleotide composition. Aptamer compositions may be double-stranded or single-stranded, and may include deoxyribonucleotides, ribonucleotides, nucleotide derivatives, or other nucleotide-like molecules.

[0045] Illustrative ligands that bind to an aptamer include, without limitation, small molecules, such as drugs, metabolites, intermediates, cofactors, transition state analogs, ions, metals, nucleic acids, and toxins. Aptamers may also bind natural and synthetic polymers, including proteins, peptides, nucleic acids, polysaccharides, glycoproteins, hormones, receptors and cell surfaces such as cell walls and cell membranes. The binding of a ligand to an aptamer, which may be DNA or RNA, causes a conformational change and alters its ability to modulate the activity of another part such as an expression platform of a riboswitch that the aptamer is part of. In one example, the conformational change inhibits translation of an mRNA in which the aptamer is located. In another example, the conformational change removes the inhibition and allows translation of an mRNA in which the aptamer is located. Aptamers may also be composed of DNA or may comprise nonnatural nucleotides and nucleotide analogs. An aptamer will most typically have been obtained by *in vitro* selection for binding of a target molecule. However, *in vivo* selection of an aptamer is also possible.

[0046] An aptamer will typically be between about 10 and about 300 nucleotides in length. More commonly, an aptamer will be between about 30 and about 100 nucleotides in length.

[0047] As used herein, the term "riboswitch", or "biosensor" refers to regulatory RNA-based biosensor that control gene expression in response to a ligand (such as luteolin), and the synthetic riboswitches developed in this disclosure can activate gene expression in response to luteolin and other naringenin derived flavonoids with high specificity. A typical riboswitch is composed of an aptamer domain and an expression platform. The term “riboswitch” reflects the ability of these noncoding RNAs to function as genetic

switches. When the abundance of a ligand (such as luteolin) exceeds a threshold level, binding of the ligand to a sensor of the riboswitch (such as the aptamer as disclosed herein) induces a conformational change of the riboswitch, leading to modulation of downstream events (e.g. in the expression platform). In one example, the RNA aptamers disclosed herein comprise a sequence that folds into a particular structure (e.g., a hairpin), which can specifically bind a ligand (such as luteolin). Binding of the ligand causes a structural change in the folding of the RNA, which induces unfolding of the hairpin and expression of the reporter gene of the expression platform.

[0048] The aptamer and the riboswitch as disclosed herein can have at least two conformational states or configurations, an "off" state and an "on" state, defined by the aptamer's ability or availability to interact with a specific ligand (such as luteolin and other naringenin derived flavonoids). For example, the aptamer can adopt a hairpin loop configuration in which the expression platform of the riboswitch is rendered unavailable to be translated, and therefore, the hairpin loop conformation can be considered as at an "off" state and the corresponding riboswitch is "off." On the other hand, a riboswitch that is "on" may have the aptamer in an "on" configuration that allows it to interact with a specific ligand (such as luteolin and other naringenin derived flavonoids). The riboswitch may switch from "off" to "on" in response to the presence of the ligand that interacts with the aptamer of the riboswitch, thereby causing a conformational change of the riboswitch that leads to the "on" configuration.

[0049] In one example, the specific ligand binding to the aptamer and riboswitch as disclosed herein is luteolin. In another example, the specific ligand binding to the aptamer and riboswitch as disclosed herein are other naringenin derived flavonoids. In one example, other naringenin derived flavonoids include apigenin. In another example, other naringenin derived flavonoids include eriodictyol.

[0050] In one example, the aptamer is a DNA aptamer. A DNA aptamer can be part of a DNA expression cassette. In another example, the aptamer is an RNA aptamer. An RNA aptamer can be part of a riboswitch.

[0051] In one example, the RNA aptamer capable of binding to luteolin and other naringenin derived flavonoids as disclosed herein comprises a nucleotide sequence of GUCCNNNNUCAGUCNNNNGACUGAGGAU (SEQ ID NO: 1). In another example, the RNA aptamer capable of binding to luteolin and other naringenin derived flavonoids as disclosed herein comprises a nucleotide sequence of

AUGCUGGNNNNNCUAGUAUNNNNACAUNNNNAUGU (SEQ ID NO: 2). In another example, the RNA aptamer capable of binding to luteolin and other naringenin derived flavonoids as disclosed herein comprises a nucleotide sequence of AAUGUUUGCAGUCCNNNNUCAGUCNNNNGACUGAGGAUAG (SEQ ID NO: 5). In another example, the RNA aptamer capable of binding to luteolin and other naringenin derived flavonoids as disclosed herein comprises a nucleotide sequence of AAUGCUGGNNNNNCUAGUAUNNNNACAUNNNNAUGUUUG (SEQ ID NO: 6). SEQ ID NO: 1 and SEQ ID NO: 5 are RNA aptamer sequence of the first biosensor of luteolin and other naringenin derived flavonoids disclosed herein (termed "**luteolin biosensor 1**" or "LB1"). SEQ ID NO: 1 is the consensus sequence, while SEQ ID NO: 5 is the full sequence of the RNA aptamer of luteolin biosensor 1. SEQ ID NO: 2 and SEQ ID NO: 6 are RNA aptamer sequence of the second biosensor of luteolin and other naringenin derived flavonoids disclosed herein (termed "**luteolin biosensor 2**" or "LB2"). SEQ ID NO: 2 is the consensus sequence, while SEQ ID NO: 6 is the full sequence of the RNA aptamer of luteolin biosensor 2. For SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 5 and SEQ ID NO: 6, N is one nucleotide selected from the group consisting of A, G, C and U.

[0052] In another example, the DNA aptamer capable of binding to luteolin and other naringenin derived flavonoids as disclosed herein comprises a nucleotide sequence of GTCCNNNNTCAGTCNNNNGACTGAGGAT (SEQ ID NO: 3). In another example, the DNA aptamer capable of binding to luteolin and other naringenin derived flavonoids as disclosed herein comprises a nucleotide sequence of ATGCTGGNNNNNCTAGTAT (SEQ ID NO: 4). In another example, the DNA aptamer capable of binding to luteolin and other naringenin derived flavonoids as disclosed herein comprises a nucleotide sequence of AATGTTTGCAGTCCNNNNTCAGTCNNNNGACTGAGGATAG (SEQ ID NO: 7). In another example, the DNA aptamer capable of binding to luteolin and other naringenin derived flavonoids as disclosed herein comprises a nucleotide sequence of AATGCTGGNNNNNCTAGTATAATCACATAACACATGTTTG (SEQ ID NO: 8). SEQ ID NO: 3 and SEQ ID NO: 7 are DNA aptamer sequence of the first biosensor of luteolin and other naringenin derived flavonoids disclosed herein (termed "**luteolin biosensor 1**" or "LB1"). SEQ ID NO: 3 is the consensus sequence, while SEQ ID NO: 7 is the full sequence of the DNA aptamer of luteolin biosensor 1. SEQ ID NO: 4 and SEQ ID NO: 8 are DNA aptamer sequence of the second biosensor of luteolin and other

naringenin derived flavonoids disclosed herein (termed "**luteolin biosensor 2**" or "LB2"). SEQ ID NO: 4 is the consensus sequence, while SEQ ID NO: 8 is the full sequence of the RNA aptamer of luteolin biosensor 2. For SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 7 and SEQ ID NO: 8, N is one nucleotide selected from the group consisting of A, G, C and T.

[0053] The **luteolin biosensor 1** as disclosed herein comprising an RNA aptamer sequence of SEQ ID NO: 1 and SEQ ID NO: 5, or a DNA aptamer sequence of SEQ ID NO: 3 and SEQ ID NO: 7 is capable of binding to luteolin or eriodictyol. The **luteolin biosensor 2** as disclosed herein comprising an RNA aptamer sequence of SEQ ID NO: 2 and SEQ ID NO: 6, or a DNA aptamer sequence of SEQ ID NO: 4 and SEQ ID NO: 8 is capable of binding to luteolin or apigenin.

[0054] Various sequences of the luteolin aptamers of the present invention are summarized in Table 3 below:

[0055] **Table 3. Sequence listing of the present disclosure**

SEQ ID NO: 1 aptamer RNA sequence of luteolin biosensor 1 (consensus sequence)	GUCCNNNNUCAGUCNNNNGACUGAGGAU
SEQ ID NO: 2 aptamer RNA sequence of luteolin biosensor 2 (consensus sequence)	AUGCUGGNNNNNCUAGUAUNNNNACAUNNNNAUGU
SEQ ID NO: 3 aptamer DNA sequence of luteolin biosensor 1 (consensus sequence)	GTCCNNNNTCAGTCNNNNGACTGAGGAT
SEQ ID NO: 4 aptamer DNA sequence of luteolin biosensor 2 (consensus sequence)	ATGCTGGNNNNNCTAGTAT
SEQ ID NO: 5 aptamer RNA sequence of luteolin biosensor 1	AAUGUUUGCAGUCCNNNNUCAGUCNNNNGACUGAGGAUAG
SEQ ID NO: 6 aptamer RNA sequence of luteolin biosensor 2	AAUGCUGGNNNNNCUAGUAUNNNNACAUNNNNAUGUUUG

SEQ ID NO: 7	AATGTTTGCAGTCCNNNNTCAGTCNNNNG
aptamer DNA sequence of luteolin biosensor 1	ACTGAGGATAG
SEQ ID NO: 8	AATGCTGGNNNNNCTAGTATAATCACATA
aptamer DNA sequence of luteolin biosensor 2	ACACATGTTTG

[0056] In another aspect, the present disclosure refers to a riboswitch comprising the RNA aptamer as disclosed herein, a ribosomal binding site (RBS) and a reporter; wherein the RNA aptamer comprises the nucleotide sequence of SEQ ID NO: 1 or SEQ ID NO: 2.

[0057] In one example, the riboswitch comprises an RNA aptamer comprising the nucleotide sequence of SEQ ID NO: 1 or SEQ ID NO: 2 being the consensus sequence of the aptamer. In another example, the riboswitch comprises an RNA aptamer comprising the nucleotide sequence of SEQ ID NO: 5 or SEQ ID NO: 6 being the full sequence of aptamer.

[0058] The RBS is selected from the group consisting of *E.coli* RBS having a consensus sequence of AGGA, a default RBS having a sequence of tttaagaaggagatatacat, and RBS34 having a sequence of tctagagaaagaggagaaatactag. In one example, the RBS is any RBS having an "AGGA" sequence.

[0059] The reporter can be any suitable reporter capable of generating a detectable signal upon conformational change of the aptamer after binding to its ligand such as luteolin and the other naringenin derived flavonoids. In one example, the reporter is a fluorescence protein. In another example, the reporter is a fluorophore. In another example, the reporter is a colour dye. In another example, the reporter is a luminophore. In another example, the reporter is luciferase.

[0060] Commonly used reporter genes are - GFP (green fluorescent protein gene), lacZ (β- galactosidase gene), RFP (red fluorescent protein gene), and Luc (luciferase gene). The cells expressing GFP glow green when excited by wavelengths of blue to the ultraviolet range, while those expressing RFP glow red when excited by wavelengths of 488 nm or 532 nm. The cells expressing the Luc gene produce luciferase enzymes that catalyze a reaction with luciferin to produce light. The lacZ gene is the most common

reporter gene used in E.coli. It produces an enzyme β-galactosidase that causes the bacteria to appear blue when grown in a media containing X-gal substrate.

[0061] Examples of the fluorescence protein include a green fluorescence protein selected from the group consisting of EGFP, Emerald, Superfolder GFP, Azami Green, mWasabi, TagGFP, TurboGFP, AcGFP, ZsGreen and T-Sapphire; a blue fluoresce protein selected from the group consisting of EBFP, EBFP2, Azurite and mTagBFP; a cyan fluoresce protein selected from the group consisting of ECFP, mECFP, Cerulean, mTurquoise, CyPet, AmCyan1, Midori-Ishi Cyan, TagCFP and mTFP1 (Teal); a yellow fluorescence protein selected from the group consisting of EYFP, Topaz, Venus, mCitrine, YPet, TagYFP, PhiYFP, ZsYellow1 and mBanana; an orange fluorescence protein selected from the group consisting of Kusabira Orange, Kusabira Orange2, mOrange, mOrange2, dTomato, dTomato-Tandem, TagRFP, TagRFP-T, DsRed, DsRed2, DsRed-Express (T1), DsRed-Monomer and mTangerine; and a red fluorescence protein selected from the group consisting of mRuby, mApple, mStrawberry, AsRed2, mRFP1, JRed, mCherry, HcRed1, mRaspberry, dKeima-Tandem, HcRed-Tandem, mPlum, and AQ143. Typical fluorescence protein reporters are listed in Table 4.

[0062] **Table 4. Examples of fluorescence protein reporters**

Protein (Acronym)	**Excitation Maximum (nm)**	**Emission Maximum (nm)**	**Molar Extinction Coefficient**	**Quantum Yield**	***in vivo* Structure**	**Relative Brightness (% of EGFP)**
GFP (wt)	395/475	509	21,000	0.77	Monomer*	48
Green Fluorescent Proteins						
EGFP	484	507	56,000	0.6	Monomer*	100
Emerald	487	509	57,500	0.68	Monomer*	116
Superfolder GFP	485	510	83,300	0.65	Monomer*	160
Azami Green	492	505	55,000	0.74	Monomer	121
mWasabi	493	509	70,000	0.8	Monomer	167
TagGFP	482	505	58,200	0.59	Monomer*	110
TurboGFP	482	502	70,000	0.53	Dimer	102
AcGFP	480	505	50,000	0.55	Monomer*	82

ZsGreen	493	505	43,000	0.91	Tetramer	117
T-Sapphire	399	511	44,000	0.6	Monomer*	79
Blue Fluorescent Proteins						
EBFP	383	445	29,000	0.31	Monomer*	27
EBFP2	383	448	32,000	0.56	Monomer*	53
Azurite	384	450	26,200	0.55	Monomer*	43
mTagBFP	399	456	52,000	0.63	Monomer	98
Cyan Fluorescent Proteins						
ECFP	439	476	32,500	0.4	Monomer*	39
mECFP	433	475	32,500	0.4	Monomer	39
Cerulean	433	475	43,000	0.62	Monomer*	79
mTurquoise	434	474	30,000	0.84	Monomer*	75
CyPet	435	477	35,000	0.51	Monomer*	53
AmCyan1	458	489	44,000	0.24	Tetramer	31
Midori-Ishi Cyan	472	495	27,300	0.9	Dimer	73
TagCFP	458	480	37,000	0.57	Monomer	63
mTFP1 (Teal)	462	492	64,000	0.85	Monomer	162
Yellow Fluorescent Proteins						
EYFP	514	527	83,400	0.61	Monomer*	151
Topaz	514	527	94,500	0.6	Monomer*	169
Venus	515	528	92,200	0.57	Monomer*	156
mCitrine	516	529	77,000	0.76	Monomer	174
YPet	517	530	104,000	0.77	Monomer*	238
TagYFP	508	524	64,000	0.6	Monomer	118
PhiYFP	525	537	124,000	0.39	Monomer*	144
ZsYellow1	529	539	20,200	0.42	Tetramer	25
mBanana	540	553	6,000	0.7	Monomer	13
Orange Fluorescent Proteins						
Kusabira Orange	548	559	51,600	0.6	Monomer	92

Kusabira Orange2	551	565	63,800	0.62	Monomer	118
mOrange	548	562	71,000	0.69	Monomer	146
mOrange2	549	565	58,000	0.6	Monomer	104
dTomato	554	581	69,000	0.69	Dimer	142
dTomato-Tandem	554	581	138,000	0.69	Monomer	283
TagRFP	555	584	100,000	0.48	Monomer	142
TagRFP-T	555	584	81,000	0.41	Monomer	99
DsRed	558	583	75,000	0.79	Tetramer	176
DsRed2	563	582	43,800	0.55	Tetramer	72
DsRed-Express (T1)	555	584	38,000	0.51	Tetramer	58
DsRed-Monomer	556	586	35,000	0.1	Monomer	10
mTangerine	568	585	38,000	0.3	Monomer	34
Red Fluorescent Proteins						
mRuby	558	605	112,000	0.35	Monomer	117
mApple	568	592	75,000	0.49	Monomer	109
mStrawberry	574	596	90,000	0.29	Monomer	78
AsRed2	576	592	56,200	0.05	Tetramer	8
mRFP1	584	607	50,000	0.25	Monomer	37
JRed	584	610	44,000	0.2	Dimer	26
mCherry	587	610	72,000	0.22	Monomer	47
HcRed1	588	618	20,000	0.015	Dimer	1
mRaspberry	598	625	86,000	0.15	Monomer	38
dKeima-Tandem	440	620	28,800	0.24	Monomer	21
HcRed-Tandem	590	637	160,000	0.04	Monomer	19
mPlum	590	649	41,000	0.1	Monomer	12
AQ143	595	655	90,000	0.04	Tetramer	11

[0063] In another aspect, the present disclosure refers to a DNA expression cassette for the regulation of the expression of the reporter as disclosed herein, comprising a promoter, a DNA aptamer as disclosed herein, a DNA sequence encoding the RBS and a DNA sequence encoding the reporter of the riboswitch as disclosed herein.

[0064] As used herein, an "DNA expression cassette" is a nucleic acid construct, generated recombinantly or synthetically, with nucleic acid elements that are capable of

effecting expression of a gene in hosts compatible with such sequences. DNA expression cassettes include at least promoters, ribosome binding site (RBS), coding sequence (CDS), and optionally, transcription termination signals. Typically, the recombinant expression cassette includes a nucleic acid to be transcribed (e.g., a nucleic acid encoding a reporter, or other protein), RBS, and a promoter. Additional factors necessary or helpful in effecting expression may also be used as described herein. For example, an expression cassette can also include DNA aptamers that modulate the expression of the reporter protein. Transcription termination signals, enhancers, and other nucleic acid sequences that influence gene expression, can also be included in an expression cassette.

[0065] Specifically, in this disclosure, the DNA expression cassette comprises the DNA aptamer as disclosed herein. In one example, the DNA expression cassette comprises the DNA aptamer comprising the nucleotide sequence of SEQ ID NO: 3 or SEQ ID NO: 4 being the consensus sequence of the aptamer. In another example, the DNA expression cassette comprises the DNA aptamer comprising the nucleotide sequence of SEQ ID NO: 7 or SEQ ID NO: 8 being the full sequence of the aptamer.

[0066] As used herein, "promoter" refers to a region of the DNA expression cassette as disclosed herein involved in binding to proteins such as RNA polymerase to initiate transcription of DNA (e.g. the reporter gene) downstream of the promoter. Promoters are located near the transcription start sites of genes, upstream on the DNA (towards the 5' region of the sense strand). Promoters can be about 30–1000 base pairs long, the sequence of which is highly dependent on the gene and product of transcription, type or class of RNA polymerase recruited to the site, and species of organism. In one example, the promoter is inducible, i.e. the promoter directs expression of a reporter gene only when receiving a specific stimulus, such as chemical, protein, light, heat, etc. In another example, the promoter is constantly on, i.e. it is always able to direct expression of a reporter gene but it is only accessible to the RNA polymerase and transcriptional factors. Subsequent translation will only be enabled when the hairpin structure of the aptamer is unfolded by binding of the ligand such as luteolin or other naringenin derived flavonoids. Promoter used herein is to enable the transcription of the DNA expression cassette, which includes the aptamer, RBS, and coding sequence (CDS). In a riboswitch, promoter controls transcription, and aptamer controls translation.

[0067] The promoter can be any promoter suitable to initiate transcription. In one example, the promoter is a *E. coli* σ^{70} promoter. In another example, the promoter is a *E.*

coli σ^{19} promoter. In another example, the promoter is a *E. coli* σ^{24} promoter. In another example, the promoter is a *E. coli* σ^{28} promoter. In another example, the promoter is a *E. coli* σ^{32} promoter. In another example, the promoter is a *E. coli* σ^{38} promoter. In another example, the promoter is a *E. coli* σ^{54} promoter. Non-exhaustive examples of the promoter are listed in Table 5. In bacteria such as *E. coli*, the promoter contains two short sequence elements approximately 10 (Pribnow Box) and 35 nucleotides upstream from the transcription start site. Sigma70 (σ^{70}) promoter comprises two well-defined short sequences located at-10 and-35 base pairs upstream of genes' transcription start sites (TSS), respectively. The sequence of the promoter at -10 (the -10 element, relative to the transcription start site) has the consensus sequence TATAAT. The sequence of the promoter at -35 (the -35 element, relative to the transcription start site) has the consensus sequence TTGACA. Optimal inter-base distance between the -10 element and the -35 element is 17(±1) bp.

[0068] **Table 5. Examples of promoter.**

pLac	IPTG inducible promoter	aattgtgagcggataacaattgacattgtgagcggataac aagatactgagcacatcagcaggacgcactgacc
PBAD	Arabinose inducible promoter	ctgacgctttttatcgcaactctctactgt
pTet	Anhydrotetracycline (ATc) incucible promoter	ttcctaatttttgttgacactctatcgttgatagagttatttac cactccctatcagtgatagagaaaa
BBa_J2 3112	P(Bla)	. . . gtttatacataggcgagtactctgttatgg
BBa_J2 3113	P(Cat)	. . . agaggttccaactttcaccataatgaaaca
BBa_J2 3114	P(Kat)	. . . taaacaactaacggacaattctacctaaca
BBa_J2 3115	Template for Building Primer Family Member	. . . acatcaagccaaattaaacaggattaacac
BBa_J2 3116	Reverse lambda cI-regulated promoter	. . . gaggtaaaatagtcaacacgcacggtgtta
BBa_J2 3117	Key Promoter absorbs 3	. . . caggccggaataactccctataatgcgcca
BBa_J2 3118	constitutive promoter family member	. . . ggctagctcagtcctaggtacagtgctagc
BBa_J2 3119	constitutive promoter family member	. . . agctagctcagtcctaggtattatgctagc
BBa_J2 3150	constitutive promoter family member	. . . agctagctcagtcctaggtactgtgctagc
BBa_J2 3151	constitutive promoter family member	. . . agctagctcagtcctagggattatgctagc
BBa_J4 4002	constitutive promoter family member	. . . agctagctcagtcctaggtattgtgctagc

BBa_J48104	constitutive promoter family member	. . . ggctagctcagtcctaggtactatgctagc
BBa_J54200	constitutive promoter family member	. . . ggctagctcagtcctaggtatagtgctagc
BBa_J56015	constitutive promoter family member	. . . ggctagctcagccctaggtattatgctagc
BBa_J64951	constitutive promoter family member	. . . agctagctcagtcctaggtataatgctagc
BBa_K088007	constitutive promoter family member	. . . agctagctcagtcctagggactgtgctagc
BBa_K119000	constitutive promoter family member	. . . ggctagctcagtcctaggtacaatgctagc
BBa_K119001	constitutive promoter family member	. . . ggctagctcagtcctaggtatagtgctagc
BBa_K1330002	constitutive promoter family member	. . . agctagctcagtcctagggattatgctagc
BBa_K137029	constitutive promoter family member	. . . ggctagctcagtcctagggattatgctagc
BBa_K137030	constitutive promoter family member	. . . ggctagctcagtcctaggtacaatgctagc
BBa_K137031	constitutive promoter family member	. . . agctagctcagcccttggtacaatgctagc
BBa_K137032	constitutive promoter family member	. . . agctagctcagtcctagggactatgctagc
BBa_K137085	constitutive promoter family member	. . . agctagctcagtcctagggattgtgctagc
BBa_K137086	constitutive promoter family member	. . . ggctagctcagtcctaggtattgtgctagc
BBa_K137087	constitutive promoter family member	. . . agctagctcagtcctaggtataatgctagc
BBa_K137088	1bp mutant from J23107	. . . ggctagctcagtcctaggtattatgctagc
BBa_K137089	1bp mutant from J23114	. . . ggctagctcagtcctaggtacaatgctagc
BBa_K137090	pBAD reverse	. . . aaagtgtgacgccgtgcaaataatcaatgt
BBa_K137091	NikR promoter, a protein of the ribbon helix-helix family of trancription factors that repress expre	. . . gacgaatacttaaaatcgtcatacttattt
BBa_K1585100	lacq_Promoter	. . . aaacctttcgcggtatggcatgatagcgcc
BBa_K1585101	lacIQ - promoter sequence	. . . tgatagcgcccggaagagagtcaattcagg
BBa_K1585102	*E. Coli* CrcABCD phosphate sensing operon promoter	. . . ttatttaccgtgacgaactaattgctcgtg
BBa_K1585103	GlnRS promoter	. . . catacgccgttatacgttgtttacgctttg

BBa_K1585104	Constitutive weak promoter of lacZ	. . . ttatgcttccggctcgtatgttgtgtggac
BBa_K1585105	Mutated LacZ promoter	. . . ttatgcttccggctcgtatggtgtgtggac
BBa_K1585106	Constitutive promoter (J23105)	. . . ggctagctcagtcctaggtactatgctagc
BBa_K1585110	constitutive promoter with (TA)10 between -10 and -35 elements	. . . atatatatatatataatggaagcgtttt
BBa_K1585113	constitutive promoter with (TA)9 between -10 and -35 elements	. . . atatatatatatataatggaagcgtttt
BBa_K1585115	constitutive promoter with (C)10 between -10 and -35 elements	. . . ccccgaaagcttaagaatataattgtaagc
BBa_K1585116	constitutive promoter with (C)12 between -10 and -35 elements	. . . ccccgaaagcttaagaatataattgtaagc
BBa_K1585117	optimized (TA) repeat constitutive promoter with 13 bp between -10 and -35 elements	. . . tgacaatatatatatatataatgctagc
BBa_K1585118	optimized (TA) repeat constitutive promoter with 15 bp between -10 and -35 elements	. . . acaatatatatatatataatgctagc
BBa_K1585119	optimized (TA) repeat constitutive promoter with 17 bp between -10 and -35 elements	. . . aatatatatatatatataatgctagc
BBa_K1824896	optimized (TA) repeat constitutive promoter with 19 bp between -10 and -35 elements	. . . tatatatatatatatataatgctagc
BBa_K2486171	optimized (TA) repeat constitutive promoter with 21 bp between -10 and -35 elements	. . . tatatatatatatatataatgctagc
BBa_K256002	optimized (A) repeat constitutive promoter with 17 bp between -10 and -35 elements	. . . aaaaaaaaaaaaaaaaatataatgctagc
BBa_K256018	optimized (A) repeat constitutive promoter with 18 bp between -10 and -35 elements	. . . aaaaaaaaaaaaaaaaatataatgctagc
BBa_K256020	Anderson Promoter with lacI binding site	. . . ggaattgtgagcggataacaatttcacaca

BBa_K256033	Anderson Promoter with lacI binding site	. . . ggaattgtgagcggataacaatttcacaca
BBa_K292000	Anderson Promoter with lacI binding site	. . . ggaattgtgagcggataacaatttcacaca
BBa_K292001	Anderson Promoter with lacI binding site	. . . ggaattgtgagcggataacaatttcacaca
BBa_K418000	Anderson Promoter with lacI binding site	. . . ggaattgtgagcggataacaatttcacaca
BBa_K418002	Anderson Promoter with lacI binding site	. . . ggaattgtgagcggataacaatttcacaca
BBa_K418003	Anderson Promoter with lacI binding site	. . . ggaattgtgagcggataacaatttcacaca
BBa_K823004	Anderson Promoter with lacI binding site	. . . ggaattgtgagcggataacaatttcacaca
BBa_K823005	Anderson Promoter with lacI binding site	. . . ggaattgtgagcggataacaatttcacaca
BBa_K823006	Anderson Promoter with lacI binding site	. . . ggaattgtgagcggataacaatttcacaca
BBa_K823007	Anderson Promoter with lacI binding site	. . . ggaattgtgagcggataacaatttcacaca
BBa_K823008	Anderson Promoter with lacI binding site	. . . ggaattgtgagcggataacaatttcacaca
BBa_K823010	Anderson Promoter with lacI binding site	. . . ggaattgtgagcggataacaatttcacaca
BBa_K823011	Anderson Promoter with lacI binding site	. . . ggaattgtgagcggataacaatttcacaca
BBa_K823013	J23100 + RBS	. . . gattaaagaggagaaatactagagtactag
BBa_K823014	A reverse complement version of BBa_J23114	. . . cattgtacctaggactgagctagccataaa
BBa_M13101	J23101:GFP	. . . caccttcgggtgggcctttctgcgtttata
BBa_M13102	J23119:IFP	. . . caccttcgggtgggcctttctgcgtttata
BBa_M13103	J23119:HO1	. . . caccttcgggtgggcctttctgcgtttata
BBa_M13104	Infrared signal reporter (J23119:IFP:J23119:HO1)	. . . caccttcgggtgggcctttctgcgtttata
BBa_M13105	Double terminator + constitutive promoter	. . . ggctagctcagtcctaggtacagtgctagc
BBa_M13106	Double terminator + Constitutive promoter + Strong RBS	. . . tgctagctactagagattaaagaggagaaa
BBa_M13108	IPTG inducible Lac promoter cassette	. . . ttgtgagcggataacaagatactgagcaca
BBa_M13110	IPTG inducible Lac promoter cassette	. . . ttgtgagcggataacaagatactgagcaca

BBa_M 31519	IPTG inducible Lac promoter cassette	. . . ttgtgagcggataacaagatactgagcaca
BBa_R1 074	Anderson promoter J23100	. . . ggctagctcagtcctaggtacagtgctagc
BBa_R1 075	Anderson promoter J23101	. . . agctagctcagtcctaggtattatgctagc
BBa_S0 3331	Anderson promoter J23102	. . . agctagctcagtcctaggtactgtgctagc
	M13K07 gene I promoter	. . . cctgtttttatgttattctctctgtaaagg
	M13K07 gene II promoter	. . . aaatatttgcttatacaatcttcctgtttt
	M13K07 gene III promoter	. . . gctgataaaccgatacaattaaaggctcct
	M13K07 gene IV promoter	. . . ctcttctcagcgtcttaatctaagctatcg
	M13K07 gene V promoter	. . . atgagccagttcttaaaatcgcataaggta
	M13K07 gene VI promoter	. . . ctattgattgtgacaaaataaacttattcc
	M13K07 gene VIII promoter	. . . gtttcgcgcttggtataatcgctgggggtc
	M13110	. . . ctttgcttctgactataatagtcagggtaa
	Modified promoter sequence of g3.	. . . aaaccgatacaattaaaggctcctgctagc
	Constitutive Promoter I	. . . caccacactgatagtgctagtgtagatcac
	Constitutive Promoter II	. . . gccggaataactccctataatgcgccacca

[0069] In another aspect, the present disclosure refers to a composition for detecting luteolin and other naringenin derived flavonoids, comprising the riboswitch as disclosed herein, and a translation mixture.

[0070] In another aspect, the present disclosure refers to a composition for detecting luteolin and other naringenin derived flavonoids, comprising the DNA expression cassette as disclosed herein, a transcription mixture and a translation mixture.

[0071] As used herein, "transcription mixture" and "translation mixture" refer to cell-free protein synthesis systems which are generally constructed with cell extract prepared from *E. coli*, wheat germ, insect cells, or rabbit reticulocytes. The *E. coli* extract-based cell-free expression system is the most popular, and broad varieties of *in vitro* protein synthesis systems are commercialized by various companies (e.g., Arbor Biosciences, Biotechrabbit, Invitrogen, Qiagen, Promega). The cell-free protein synthesis system has several advantages over *in vivo* processes. Firstly, since there is no need to support cellular metabolism; all of the cellular resources can be efficiently directed toward the production of an encoded protein. Although the coupled (i.e., combining both transcription and translation processes) cell-free system has been proven to be more efficient than the uncoupled one, it is possible to use mRNA or PCR fragments as the matrices escaping genetic engineering and cloning procedures. Taken together, this makes cell-free

technology a reliable and fast way to obtain a high yield of the desired protein. Secondly, the reaction environment can be directly controlled and easily manipulated, since there is no cell barrier. In addition, the cell-fee reaction mixture can be supplemented with the required additives. These additives include chaperones and reducing agents, stabilizing mRNA or promoting complex protein folding, hydrophobic compounds required for soluble expression of membrane proteins (MPs), rare tRNA molecular species for codon usage bias compensation, and orthogonal aminoacyl-tRNA synthetase (aaRS)/tRNA pairs (orthogonal translation system, OTS) for site-specific non-canonical amino acids (NAAs) incorporation in response to unique codon.

[0072] Any *E. coli* cell-free transcription or translation mixture (lab-made or commercially available mixture, including but not limited to Promega, NEB, myTXTL Sigma 70 Master Mix, Arbor Science, can be used in the composition as disclosed herein. The composition as disclosed herein facilitates the functioning of the riboswitch as disclosed herein or the DNA expression cassette as disclosed herein for the detection of luteolin and the other naringenin derived flavonoids *in vitro*.

[0073] As used herein, "*in vitro*" detection of luteolin and the other naringenin derived flavonoids refers to detecting luteolin and the other naringenin derived flavonoids outside a host cell (such as a microbe) producing the luteolin and the other naringenin derived flavonoids.

[0074] In another aspect, the present disclosure refers to a microbe containing the riboswitch as disclosed herein or the DNA expression cassette as disclosed herein.

[0075] In one example, the microbe is a prokaryote. The prokaryote can be a bacterium, or an archacon. The prokaryote is selected from the group consisting of *E. coli*, *B. subtilis*, Pseudomonas, Lactobacillus, Spirilla, Vibrio, Salmonella, Acetobacter, Cyanobacteria, Spirochetes, Chlamydias, Caulobacter, Mycoplasma, Aliivibrio, Bacteroides, Synechocystis, Azotobacter, Streptomyces, Thermococcus, Sulfolobus, Streptococcus, Methanococcus, Halobacterium, other proteobacteria, and a gram-positive bacterium. In another example, the microbe is *E. coli* 10-beta.

[0076] In another aspect, the present disclosure refers to a method of detecting luteolin and other naringenin derived flavonoids produced in a microbe, comprising (a) introducing the riboswitch as disclosed herein or the DNA expression cassette as disclosed herein into the microbe, wherein when the microbe produces the luteolin and the other naringenin derived flavonoids in an effective amount, the luteolin and the other

naringenin derived flavonoids specifically bind the aptamer to induce a conformational change in the riboswitch that allows translation of the reporter into a reporter protein; and (b) detecting the reporter protein, wherein the presence of the reporter protein is indicative of detection of the luteolin and other naringenin derived flavonoids.

[0077] The method as disclosed above refers to "*in vivo*" detection of luteolin and the other naringenin derived flavonoids produced inside a host cell (such as a microbe as disclosed herein). As used herein, "*in vivo*" detection refers to detecting luteolin and the other naringenin derived flavonoids inside a host cell (such as a microbe) that produces the luteolin and the other naringenin derived flavonoids.

[0078] For step (a) of the above "*in vivo*" detection method, the riboswitch as disclosed herein or the DNA expression cassette as disclosed herein can be introduced (or transfected) into the microbe producing luteolin and other naringenin derived flavonoids. In one example, other naringenin derived flavonoids include apigenin. In another example, other naringenin derived flavonoids include eriodictyol. Transfection may occur under natural or artificial conditions according to various methods well known in the art, and may rely on any known method for the insertion of the riboswitch as disclosed herein or the DNA expression cassette as disclosed herein into a prokaryotic microbe disclosed herein. The method for transfection is selected based on the type of prokaryotic microbe being transfected and may include, but is not limited to, bacteriophage or viral infection, electroporation, heat shock, lipofection, nanoparticle delivery, and particle bombardment. The term "transfected cells/transformed cells" includes stably transfected/transformed cells in which the riboswitch as disclosed herein or the DNA expression cassette as disclosed herein is capable of replication either as an autonomously replicating plasmid or as part of the host chromosome, as well as transiently transformed cells which express the introduced riboswitch as disclosed herein or the DNA expression cassette as disclosed herein for limited periods of time.

[0079] In the above "*in vivo*" detection method, once the riboswitch as disclosed herein or the DNA expression cassette as disclosed herein is introduced into the microbe, the DNA expression cassette is transcribed into the riboswitch containing the aptamer disclosed herein. When the microbe produces the luteolin and the other naringenin derived flavonoids in an effective amount, the luteolin and the other naringenin derived flavonoids specifically bind the aptamer of the riboswitch as disclosed herein or the DNA

expression cassette as disclosed herein, and this binding induces a conformational change in the riboswitch that allows translation of the reporter into a reporter protein.

[0080] The presence of the reporter protein is indicative of detection of the luteolin and other naringenin derived flavonoids. Detecting the reporter protein is done by a method selected from the group consisting of fluorescent detection, colorimetric detection, bioluminescence detection, spectrophotometry, and DNA microarray.

[0081] In another aspect, the present disclosure refers to a method of detecting luteolin and other naringenin derived flavonoids produced by a microbe, comprising (a) incubating the microbe with the composition as disclosed herein in a medium, wherein when the microbe produces and releases the luteolin and the other naringenin derived flavonoids in an effective amount into the medium, the luteolin and the other naringenin derived flavonoids specifically bind the aptamer of the riboswitch to induce a conformational change in the riboswitch that allows translation of the reporter into a reporter protein; and (b) detecting the reporter protein, wherein the presence of the reporter protein is indicative of detection of the luteolin and other naringenin derived flavonoids.

[0082] The method as disclosed above is the first embodiment of "*in vitro*" detection of luteolin and the other naringenin derived flavonoids produced by a host cell (such as a microbe as disclosed herein). As used herein, "*in vitro*" detection of luteolin and the other naringenin derived flavonoids refers to detecting luteolin and the other naringenin derived flavonoids outside a host cell (such as a microbe) which produces the luteolin and the other naringenin derived flavonoids.

[0083] For step (a) of the first embodiment of "*in vitro*" detection method as disclosed above, (i) the composition comprising the riboswitch as disclosed herein and a translation mixture; or (ii) the composition comprising the DNA expression cassette as disclosed herein, a transcription mixture and a translation mixture, is incubated in a medium, in which the microbe producing the luteolin and the other naringenin derived flavonoids is also incubated. When the microbe produces and releases the luteolin and the other naringenin derived flavonoids in an effective amount into the medium, the luteolin and the other naringenin derived flavonoids specifically bind the aptamer of the riboswitch (the DNA expression cassette can be transcribed into the riboswitch containing the aptamer disclosed herein, by the transcription mixture included in the composition), to induce a conformational change in the riboswitch that allows translation of the reporter

into a reporter protein, using the translation mixture included in the composition disclosed herein. In one example, other naringenin derived flavonoids include apigenin. In another example, other naringenin derived flavonoids include eriodictyol.

[0084] Any *E. coli* cell-free transcription or translation mixture (lab-made or commercially available mixture, including but not limited to Promega, NEB, myTXTL Sigma 70 Master Mix, Arbor Science, can be used in the composition as disclosed herein. The composition as disclosed herein facilitates the functioning of the riboswitch as disclosed herein or the DNA expression cassette as disclosed herein for the detection of luteolin and the other naringenin derived flavonoids *in vitro*.

[0085] The presence of the reporter protein is indicative of detection of the luteolin and other naringenin derived flavonoids. Detecting the reporter protein is done by a method selected from the group consisting of fluorescent detection, colorimetric detection, bioluminescence detection, spectrophotometry, and DNA microarray.

[0086] In another aspect, the present disclosure refers to a method of detecting luteolin and other naringenin derived flavonoids produced by a first microbe, comprising (a) incubating the first microbe and the microbe as disclosed herein (containing the riboswitch or DNA expression cassette) in a medium; wherein when the first microbe produces and releases the luteolin and the other naringenin derived flavonoids in an effective amount into the medium, the luteolin and the other naringenin derived flavonoids specifically bind the aptamer of the riboswitch produced by the microbe as disclosed herein to induce a conformational change in the riboswitch that allows translation of the reporter into a reporter protein; and (b) detecting the reporter protein, wherein the presence of the reporter protein is indicative of detection of the luteolin and the other naringenin derived flavonoids.

[0087] The method as disclosed above is the second embodiment of "*in vitro*" detection of luteolin and the other naringenin derived flavonoids produced by a host cell (such as a microbe as disclosed herein). As used herein, "*in vitro*" detection of luteolin and the other naringenin derived flavonoids refers to detecting luteolin and the other naringenin derived flavonoids outside a host cell (such as a microbe) which produces the luteolin and the other naringenin derived flavonoids.

[0088] For step (a) of the second embodiment of "*in vitro*" detection method as disclosed above, a first microbe producing the luteolin and the other naringenin derived flavonoids is co-cultured with a second microbe which has been transformed/transfected

with riboswitch as disclosed herein or the DNA expression cassette as disclosed herein. When the first microbe produces and releases the luteolin and the other naringenin derived flavonoids in an effective amount into the medium, the luteolin and the other naringenin derived flavonoids specifically binds the aptamer of the riboswitch produced by the transformed/transfected second microbe to induce a conformational change in the riboswitch that allows translation of the reporter into a reporter protein. In one example, other naringenin derived flavonoids include apigenin. In another example, other naringenin derived flavonoids include eriodictyol.

[0089] The presence of the reporter protein is indicative of detection of the luteolin and other naringenin derived flavonoids. Detecting the reporter protein is done by a method selected from the group consisting of fluorescent detection, colorimetric detection, bioluminescence detection, spectrophotometry, and DNA microarray.

[0090] In the "*in vitro*" detection method or "*in vivo*" detection method as disclosed herein, the microbe producing the luteolin and the other naringenin derived flavonoids, or the microbe which has been transformed/transfected with riboswitch as disclosed herein or the DNA expression cassette as disclosed herein, is a prokaryote. The prokaryote can be a bacterium, or an archaeon. The prokaryote is selected from the group consisting of *E. coli*, *B. subtilis*, Pseudomonas, Lactobacillus, Spirilla, Vibrio, Salmonella, Acetobacter, Cyanobacteria, Spirochetes, Chlamydias, Caulobacter, Mycoplasma, Aliivibrio, Bacteroides, Synechocystis, Azotobacter, Streptomyces, Thermococcus, Sulfolobus, Streptococcus, Methanococcus, Halobacterium, other proteobacteria, and a gram-positive bacterium. In another example, the microbe is *E. coli* 10-beta. The biosensors as disclosed herein maintain their stability and reproducibility in detecting luteolin and other naringenin derived flavonoids when transfected/transformed in various microbes as disclosed herein.

[0091] In the "*in vitro*" detection method or "*in vivo*" detection method as disclosed herein, a range of suitable media are available for incubating the microbe as disclosed herein, the types and compositions of which are well known to those of skill in the art. Preferably the culture medium contains at least water, salts, nutrients, essential amino acids, vitamins and hormones, and may also include one or more growth factors. A variety of suitable culture media is commercially available, for example LB broth, LB Agar, Terrific Broth, M9, M63, SOC Medium, ImMedia Medium, MagicMedia Medium. In one example, the culture medium is LB broth medium. In another example, the culture

medium is M9 medium. The biosensors as disclosed herein maintain their stability and reproducibility in detecting luteolin and other naringenin derived flavonoids, in various culture media as disclosed herein, such as LB broth medium, or M9 medium.

[0092] The biosensors as disclosed herein maintain their stability and reproducibility in detecting luteolin and other naringenin derived flavonoids at different temperatures, from 18-37 °C, for example, 18-25 °C, 25-30 °C, 30-37 °C, about 18 °C, about 19 °C, about 20 °C, about 21 °C, about 22 °C, about 23 °C, about 24 °C, about 25 °C, about 26 °C, about 27 °C, about 28 °C, about 29 °C, about 30 °C, about 31 °C, about 32 °C, about 33 °C, about 34 °C, about 35 °C, about 36 °C, and about 37 °C.

[0093] Using the "*in vitro*" detection method or "*in vivo*" detection method as disclosed herein, luteolin and the other naringenin derived flavonoids can be detected at a concentration of at least 1 μM, at least 1.75 μM, at least 2 μM, at least 2.25 μM, at least 5 μM, at least 7.5 μM, at least 10 μM, at least 15 μM, at least 17.5 μM, at least 20 μM, at least 25 μM, at least 27.5 μM, at least 30 μM, at least 35 μM, at least 70 μM, at least 105 μM, at least 110 μM, at least 115 μM, at least 120 μM, or a higher concentration. In some examples, using the "*in vitro*" detection method or "*in vivo*" detection method as disclosed herein, luteolin and the other naringenin derived flavonoids can be detected at a concentration of about 1 μM, about 1.75 μM, about 2 μM, about 2.25 μM, about 5 μM, about 7.5 μM, about 10 μM, about 15 μM, about 17.5 μM, about 20 μM, about 25 μM, about 27.5 μM, about 30 μM, about 35 μM, about 70 μM, about 105 μM, about 110 μM, about 115 μM, about 120 μM, or a higher concentration. The level of the reporter protein translated is at least about 1.24-fold higher when luteolin and the other naringenin derived flavonoid is present than the level when luteolin and the other naringenin derived flavonoid is absent. In some examples, the level of the reporter protein translated is at least about 1.24-fold, about 2-fold, about 2.8-fold, about 3-fold, about 3.1-fold, about 4-fold, about 4.46-fold, about 5-fold, about 5.12-fold, about 5.4-fold, about 6-fold, about 6.56-fold, about 7-fold, about 7.4-fold, about 7.55-fold, about 7.72-fold, about 8-fold, about 8.11-fold, about 8.18-fold, about 9-fold, and about 9.6-fold higher when luteolin and the other naringenin derived flavonoid is present than the level when luteolin and the other naringenin derived flavonoid is absent.

[0094] As such, the aptamers and riboswitches in the present disclosure enable ultra-high throughput and highly sensitive, specific, and effective screening of producer strains of luteolin and the other naringenin derived flavonoids.

[0095] In certain embodiments, the disclosure provides a method of co-detecting eriodictyol and apigenin produced by a microbe or microbes. The **luteolin biosensor 1** as disclosed herein comprising a RNA aptamer sequence of SEQ ID NO: 1 and SEQ ID NO: 5, or a DNA aptamer sequence of SEQ ID NO: 3 and SEQ ID NO: 7 is capable of binding to luteolin or eriodictyol. The **luteolin biosensor 2** as disclosed herein comprising a RNA aptamer sequence of SEQ ID NO: 2 and SEQ ID NO: 6, or a DNA aptamer sequence of SEQ ID NO: 4 and SEQ ID NO: 8 is capable of binding to luteolin or apigenin. Both luteolin biosensor 1 and luteolin biosensor 2 can be used to detect eriodictyol and apigenin together. To co-detect eriodictyol and apigenin, the **luteolin biosensor 1** comprises a first reporter for detecting eriodictyol, and the **luteolin biosensor 2** comprises a second reporter different from the first reporter for detecting apigenin.

[0096] As used in this application, the singular form "a," "an," and "the" include plural references unless the context clearly dictates otherwise. For example, the term "a primer" includes a plurality of primers, including mixtures and combinations thereof.

[0097] As used herein, the term "comprising" means "including." Variations of the word "comprising", such as "comprise" and "comprises," have correspondingly varied meanings. Thus, for example, a composition "comprising" X may consist exclusively of X or may include one or more additional unrecited components.

[0098] As used herein, the term "about" in the context of concentration of a substance, size of a substance, length of time, or other stated values means +/- 5% of the stated value, or +/- 4% of the stated value, or +/- 3% of the stated value, or +/- 2% of the stated value, or +/- 1% of the stated value, or +/- 0.5% of the stated value.

[0099] Throughout this disclosure, certain embodiments may be disclosed in a range format. It should be understood that the description in range format is merely for convenience and brevity and should not be construed as an inflexible limitation on the scope of the disclosed ranges. Accordingly, the description of a range should be considered to have specifically disclosed all the possible sub-ranges as well as individual numerical values within that range. For example, description of a range such as from 1 to 6 should be considered to have specifically disclosed sub-ranges such as from 1 to 3, from 1 to 4, from 1 to 5, from 2 to 4, from 2 to 6, from 3 to 6 etc., as well as individual numbers within that range, for example, 1, 2, 3, 4, 5, and 6. This applies regardless of the breadth of the range.

[00100] The invention illustratively described herein may suitably be practiced in the absence of any element or elements, limitation or limitations, not specifically disclosed herein. Thus, for example, the terms "comprising", "including", "containing", etc. shall be read expansively and without limitation. Additionally, the terms and expressions employed herein have been used as terms of description and not of limitation, and there is no intention in the use of such terms and expressions of excluding any equivalents of the features shown and described or portions thereof, but it is recognized that various modifications are possible within the scope of the invention claimed. Thus, it should be understood that although the present invention has been specifically disclosed by preferred embodiments and optional features, modification and variation of the inventions embodied herein disclosed may be resorted to by those skilled in the art, and that such modifications and variations are considered to be within the scope of this invention.

[00101] The invention has been described broadly and generically herein. Each of the narrower species and subgeneric groupings falling within the generic disclosure also form part of the invention. This includes the generic description of the invention with a proviso or negative limitation removing any subject matter from the genus, regardless of whether or not the excised material is specifically recited herein.

[00102] Unless otherwise indicated, all technical and scientific terms used herein have the same meaning as commonly understood to one of ordinary skill in the art to which the invention belongs.

[00103] Other embodiments are within the following claims and non-limiting examples.

EXAMPLES

[00104] Non-limiting examples of the disclosure will be further described in greater detail by reference to specific Examples, which should not be construed as in any way limiting the scope of the disclosure.

[00105] **Example 1 – Material and Methods**

[00106] ***1.1 Preparation of luteolin-beads conjugation***

[00107] First 1 L dilute H_2SO_4 was diluted to pH 6 in DI water. Add 50 µl of well-mixed BcMag Long-Arm Amine Terminated Magnetic Beads (Bioclone, USA) to an Eppendorf tube. Then 4 µl of 35 µM luteolin (≥98 %,Sigma, USA), was added (or appropriate ketone-containing target compound) to 996 µl of dilute H_2SO_4 (pH 6) (Sigma,

USA) to make a solution of 140 nM luteolin in diluted acid. The luteolin-acid solution was dispensed to 96-well plate, with 300 µl in each well, and the samples were prepared in triplicates. After which, the absorbance of the samples was read at 355 nm. After reading the absorbance, 300 µl samples were recovered and added to the magnetic beads. The beads and solution were mixed and incubated for 3 h, with shaking (250 rpm) for 1 h. After incubation, the absorbance of the supernatant was measured at 355 nm to confirm that the concentration of free luteolin has decreased. The beads were then washed with 1 ml PBS (phosphate-buffered saline) solution (1st Base, Singapore) to remove all unbound luteolin from the beads. A magnet was used to aggregate and separate the beads before aspirating the wash solution for discarding. After discarding the third wash solution, the beads were resuspended in 1 ml PBS and stored at 4 °C.

[00108] ***1.2. In vitro selection of the aptamers***

[00109] In this work, luteolin aptamers were selected and amplified using SELEX workflow [55]. The single-stranded oligos library was prepared by heating at 95 °C for 10 min, and subsequently added to the luteolin-bound magnetic beads. After incubating for an hour at 37 °C with gentle shaking, the mixture was exposed to a magnet to isolate the beads from the unbound oligos. The beads were washed using PBS, and the bound oligos on the beads were then amplified through PCR (KOD One™ PCR Master Mix, TOYOBO, Japan) for 30 cycles. In this study, 30 cycles of PCR at initial rounds provided an adequate amount of aptamers for subsequent rounds of SELEX. The PCR product was run through gel electrophoresis to confirm the size of the oligos. Gel extraction was then performed to extract and purify the oligos. PCR product was heated to form single-stranded oligos for next round of SELEX. Fifteen rounds of SELEX were conducted before terminating the selection.

[00110] ***1.3. Development of riboswitches***

[00111] The selected and enriched PCR product was cloned into the vector pBbE6k, a gift from Jay Keasling (Addgene plasmid #35288; http://n2t.net/addgene:35288; RRID:Addgene_35288 [56]). The vector was replaced with chloramphenicol resistance gene. The pLac promoter was replaced by Anderson promoter J23114 to drive the transcription. The amplified oligos were cloned onto upstream of GFP gene, which served as reporter. The constructed riboswitches were transformed into E. coli Acella. The transformants were spread onto the LB agar (Thermo Fisher Scientific, USA) plate without luteolin. Subsequently, the colonies formed on the plate were replicated onto a

new LB agar plate with 35 μM luteolin [57]. After overnight incubation, the fluorescence of the colonies on both plates were compared under an illuminator, and the colonies with more significant fluorescence intensity change were selected for characterization.

[00112] ***1.4. Characterization of dose-response of the developed riboswitches***

[00113] Cells were inoculated from glycerol stock and incubated overnight in LB (Lysogeny broth) supplemented with 25 μg/ml chloramphenicol at 37 °C with shaking at 225 rpm. Subsequently, 50 μl of the overnight culture was transferred into 5 ml fresh LB (Thermo Fisher Scientific, USA) containing 25 μg/ml chloramphenicol (Sigma, USA) and incubated for 2 h. The optical density at 600 nm (OD600) was measured, and the culture was then diluted to an OD600 of 0.1 using fresh LB supplemented with 25 μg/ml chloramphenicol. Based on the usual concentrations in microbial production systems [58], flavonoids were added to final concentrations of 17.5, 35, 70, and 105 μM, respectively. The mixtures were dispensed into a 96-well plate (Greiner Bio-One, Austria) with 300 μl in each well. Samples without flavonoids served as controls. Each sample was prepared in triplicate. Cell growth (measured as absorbance at 600 nm) and biosensor response (quantified by GFP fluorescence with excitation and emission wavelengths of 470 nm and 520 nm, respectively, using a gain of 75) were monitored using a microplate reader (H1, Biotek, USA). The 96-well plate was shaken kinetically, and readings were taken every 10 min over a period of 24 h. All data were baseline-corrected using the reading of LB supplemented with 25 μg/ml chloramphenicol.

[00114] ***1.5. HPLC for luteolin and other flavonoids measurement***

[00115] Naringenin (≥98 %, Sigma), apigenin (≥97 %, Sigma), eridictyol (≥98 %, Sigma), and luteolin were detected and quantified using high performance liquid chromatography (HPLC) (Hitachi, Japan). First, the standard curve for the flavonoids were established and shown in Fig. 6. As for sample preparation, 1 ml of E. coli cultures were mixed with 1 ml of ethanol (≥99.5 %, Sigma, USA) and mixed gently with pipetting. Mixture was filtered through a Acrodisc 25 mm w/0.2μm filter (PALL, USA) using a syringe. Filtrate was analysed with a Chromaster HPLC (Hitachi, Japan) equipped with a Hitachi C18 column (4.5 mmI.D x 150 mmL, 5 μM), with an injection volume of 10 μl, column temperature of 40 °C and a flow rate of 1.5 ml/min for 30 min. Mobile phase of HPLC consists of 40 % methanol (≥99.9 %, Sigma) and 6 % acetic acid (≥ 99.7 %, Sigma-Aldrich) solution dissolved in water. Signals for all compounds were detected at 300 nm.

[00116] ***1.6. Construction of the luteolin-producing E. coli strain***

[00117] Plasmid pE6k-FNS to produce luteolin from eridictyol catalysed by the enzyme flavone synthase I (Accession number: Q7XZQ8.1) was assembled with Hi-Fi Gibson Assembly (NEB, UK). DNA sequence for FNS I was synthesized by IDT (Integrated DNA Technologies, USA). The FNS gene was cloned onto downstream of the IPTG-inducible promoter in the plasmid vector pBbE6k-RFP, and the RFP gene was removed. pBbE6k-RFP was a gift from Jay Keasling (Addgene, plasmid #35288;). Assembled plasmids were transformed via heat shock into Acella E. coli strain and recovered in LB media. Primers used in the study is shown in Table 6.

[00118] **Table 6. Primers used in the present work**

Primers	**Oligo sequence (5'-3')**
FNS FWD (1)	tttaagaaggagatatacatatggctccgaccactatcac
E6K REV (1)	gtgatagtggtcggagccatatgtatatctccttcttaaa
E6K FWD (1)	gcgctgatgaaaacttagctttaaggatccaaactcgagt
FNS REV (1)	actcgagtttggatccttaaagctaagttttcatcagcgc
pAC FWD	ggctagctcagtcctaggtacaatgctagctggggacgacgatgacacga
pAC REV	cattgtacctaggactgagctagccataaacactgaaatctagaaatatt
pAC-Cam FWD	tgcgaatatatgtgtagaaactgccggaaatcgtcg
pAC-Cam REV	cgacgatttccggcagtttctacacatatattcgca

[00119] ***1.7. Sensing of intracellular luteolin produced by E. coli***

[00120] The luteolin-producing plasmid pE6K-FNS and luteolin biosensor were co-transformed into E. coli Acella. Cells were inoculated from glycerol stock and incubated overnight in LB supplemented with 50 µg/ml kanamycin (Sigma, USA) and 25 µg/ml chloramphenicol at 37 °C with shaking at 225 rpm. Following this, 50 µl of the overnight culture was transferred into 5 ml fresh LB and incubated for 2 h. The cell density was measured, and the culture was then diluted to an OD600 of 0.1 using fresh LB. A gradient of IPTG (Sigma, USA) concentrations of 0, 50, 100, 200, 300 µM was set, while the eriodictyol concentration was fixed at 105 µM. The mixtures were dispensed into a 96-well plate (Greiner Bio-One, Austria) with 300 µl in each well, and each sample was prepared in triplicate. Cell growth and GFP fluorescence were measured over 24 h using

the microplate reader. At the endpoint, the cell culture in the well plate was collected directly for HPLC analysis to determine the concentration of luteolin produced. The correlation between GFP reading and luteolin produced by E. coli was analysed.

[00121] **Example 2 – Results and discussion**

[00122] ***3.1 Selection of aptamers for luteolin***

[00123] SELEX is a well-established method for selecting oligonucleotide sequences that can bind to a target molecule with high affinity and specificity. The process involves multiple rounds of incubation and PCR amplification to isolate and amplify the oligos that bind to luteolin. In this work, luteolin aptamers were selected and amplified using SELEX. Luteolin-binding aptamers were first selected and enriched in vitro using SELEX process, as outlined in Fig. 1A. Luteolin was first conjugated to the beads, and single-stranded oligos library was added for binding. Bound oligos were amplified via PCR, and the purified PCR product was then denatured to generate a single-stranded oligos library for next round of SELEX. This process allowed for the enrichment of luteolin-binding aptamers. To enable luteolin quantification, the absorbance spectrum and the standard curve of luteolin was established (Fig. 7 and Fig. 8). Successful conjugation of luteolin to the beads was confirmed by measuring the absorbance of the supernatant before and after binding. Fig. 1B shows that, the majority (90 %) of luteolin was immobilized at room temperature, demonstrating efficient conjugation. Additionally, stability studies showed that the conjugated luteolin exhibit minimal release into the supernatant during the SELEX rounds and after three months of storage at 4 °C (Fig. 9), confirming that luteolin-conjugated beads remained stable. The SELEX process was monitored by evaluating PCR yield across rounds. Fig. 1C shows that the PCR yield increased with additional SELEX rounds, implying enrichment of luteolin-binding aptamers. The yield of PCR saturated between SELEX rounds 13–15, suggesting that most oligos at that stage were able to bind the beads. As a result, SELEX was terminated at round 15.

[00124] ***1.2 Development of synthetic luteolin riboswitches***

[00125] Next, luteolin riboswitches were constructed and selected using the aptamers enriched through SELEX. The aptamers were assembled into plasmids and cloned into the E. coli cells, with green fluorescence protein (GFP) serves as the reporter for riboswitches activity (experiment was conducted to ensure the GFP signal was not interfered by the flavonoids' auto-fluorescence, Fig. 10). In the absence of luteolin, the riboswitch would likely form a secondary structure that blocks the Ribosome Binding

Site (RBS) and inhibits translation. Upon luteolin binding, the riboswitch undergoes a conformational change that exposes RBS and enables translation of GFP, as illustrated in Fig. 2A. In other words, in the presence of luteolin, the cell harboring luteolin riboswitch would produce GFP as the readout.

[00126] To select functional riboswitches, luteolin concentration of 35 μM was used, as most recombinant E. coli strains produce luteolin below this concentration [13]. Chloramphenicol (25 μg/ml) was used to select and maintain cells harboring riboswitch plasmids. Since the biosensors were designed to operate effectively in LB medium, to minimize differences in interfering factors and ensure consistency in the biosensors' operating conditions, the replica method utilized LB agar that sourced from the same supplier as the LB broth medium. After transformation, colonies were replicated onto another agar plate with 35 μM luteolin. Functional riboswitches were found by comparing the change in GFP between the two plates (illustrated in Fig. 11). In this work, two luteolin riboswitches were identified, named Luteolin Biosensor 1 (LB1) and Luteolin Biosensor 2 (LB2). The sensing element of the LB1 is the aptamer sequence "AATGTTTGCAGTCCNNNNTCAGTCNNNNGACTGAGGATAG" (RNA aptamer: AAUGUUUGCAGUCCNNNNUCAGUCNNNNGACUGAGGAUAG). The sensing element of the LB2 is is "AATGCTGGNNNNNCTAGTATAATCACATAACACATGTTTG" (RNA aptamer: AAUGCUGGNNNNNCUAGUAUNNNNACAUNNNNAUGUUUG). Their functionality was subsequently assessed in liquid LB culture through continuous incubation and GFP measurement using a spectrophotometer. The results indicated that a 16 h incubation period was sufficient to observe differences in GFP expression levels (Fig. 2B, Fig. 12). When 35 μM luteolin was added, LB1 and LB2 showed ~3.9 and ~4.6-fold activation respectively, confirming their responsiveness to luteolin. Interestingly, structural analysis revealed that the aptamers in both LB1 and LB2 retained similar conformations in both DNA and RNA forms (Fig. 13). This finding suggests that it is possible to use DNA oligos from SELEX for riboswitches development, reducing in vitro transcription and reverse-transcription steps.

[00127] ***2.3 Dose-responses of the developed biosensors***

[00128] To evaluate the performance of the developed biosensors, dose-response experiments were conducted across various luteolin concentrations. The concentrations gradient was set at 0, 1.75, 17.5, 35, 70, and 105 μM, by taking together the consideration

of luteolin concentration reported in microbial production systems [13, 57], physiologically relevant flavonoid levels [58], and luteolin solubility. The biosensor activity was quantified by GFP intensity measured using a microplate reader.

[00129] Figure 3A and 3B show the dose-response curves for LB 1 and LB 2, respectively, based on GFP/OD reading after 16 hours of incubation. The fold change between 0 and 105 μM for LB1 and LB2 was around eight. The dose-response curves were fitted using Hill equation, with $R^2 > 0.97$ for both LB1 and LB2. Both LB1 and LB2 showed activation at low luteolin concentration (1.75 μM), with fold changes of 1.48 and 1.24-fold change ($p < 0.01$), respectively. LB1 showed a lower EC_{50} (11.71 μM) and a greater fold change at 1.75 μM compared to LB2 (Figure 14), indicating higher affinity to luteolin and faster saturation. In contrast, LB2 exhibited a higher EC_{50} and a more pronounced increase in GFP expression at higher luteolin concentrations, reflecting a broader dynamic range. Besides, LB2 has steeper slope, suggesting LB2 has higher sensitivity than LB1. The time-response profiles of LB1 and LB2 (Figure 15) revealed a sharp increase within the first 50-60 minutes, demonstrating the rapid response of the luteolin biosensors. Further, the stability and reproducibility of the biosensors were validated by characterizing them in a different host, *E. coli* 10-beta (Figure 16), and in a different culture medium, M9 (Figure 17), where they maintained their ability to detect luteolin. These findings shows that riboswitch-based biosensors performed well under a wide range of conditions.

[00130] ***2.4 Characterizing the response of developed biosensors to other flavonoids***

[00131] In the process of luteolin production, metabolic pathways involve a range of enzymatic reactions where flavonoids might act as substrates or products (Figure 18A). It is important for the biosensor to be able to distinguish the target products from the substrates. Therefore, the responses of the biosensors to three other flavonoids, specifically apigenin, eriodictyol, and naringenin, was evaluated. Naringenin usually serves as the substrate for flavonoids synthesis, while apigenin and eriodictyol can also serve as intermediates in luteolin synthesis.

[00132] Fluorescence intensities of the biosensors were measured at flavonoid concentrations of 0, 17.5, 35, 70, and 105 μM. The dose-responses of LB1 to different flavonoids were shown in Figure 4A. The results showed that the GFP fold activation (normalized to GFP expression at 0 μM flavonoid) of LB1 increased with increasing luteolin and eriodictyol concentrations, taking note that the response of LB1 to luteolin

was 2.4-fold higher ($p < 0.001$) than eriodictyol at 70 μM. More importantly, the response to luteolin was 4 times higher than naringenin at concentration of 70 μM, implying that LB1 is not sensitive towards naringenin. In addition, while LB1 showed minimal response to apigenin, with no clear increasing trend, suggesting low sensitivity or saturation at low apigenin concentrations.

[00133] Figure 4B presents the fold activation of LB2 to different flavonoids. Overall, LB2 showed stronger GFP expression, with 3.5-fold higher fold activation at 70 μM luteolin compared to LB1. LB2 displayed an increasing trend of GFP expression in respond to higher luteolin and apigenin concentrations. However, the response to luteolin was much stronger than apigenin (2.2-fold higher GFP expression at 70 μM), implying that LB2 is more specific towards luteolin. In contrast, LB2 showed no increasing trend for eriodictyol, suggesting the biosensors might have saturated at low concentration, or poorly respond to eridictyol. Moreover, response of LB2 to luteolin was 5.2 times higher than naringenin, suggesting very low specificity to naringenin.

[00134] To further quantify specificity, fluorescence intensities for each flavonoid were normalized against the response to luteolin at the same concentration (Figure 4C). Both LB1 and LB2 were more responsive to luteolin as compared to apigenin, eriodictyol, and naringenin, despite structural similarities among the flavonoids (illustrated in Figure 4C). While eriodictyol might have some interference with LB2 at lower concentration such as 17.5μM, both LB1 and LB2 maintained stronger response to luteolin than other flavonoids at higher concentrations. Based on the observations, the biosensors' utility in flavonoid detection extends beyond luteolin. For example, results in Figure 5 demonstrated that the LB1's readout aligned very well with HPLC measurements of luteolin concentration produced by *E. coli,* even in the presence of eriodictyol. Furthermore, the biosensors can be applied to monitor other flavonoids by carefully selecting the biosensors and the synthesis pathways. For instance, when converting naringenin to eriodictyol or apigenin (Figure 18A), LB1 can be used for eriodictyol and LB2 for apigenin, respectively. The application of using LB2 for sensing apigenin production from naringenin was further demonstrated in Figure 19, highlighting versatility of the biosensors in various applications.

[00135] ***2.5 Sensing intracellular E. coli-produced luteolin***

[00136] In addition to the ability of LB1 and LB2 to detect extracellular flavonoids, the intracellular sensing capabilities of the developed luteolin biosensors was further

demonstrated. To this end, a luteolin-producing *E. coli* strain was engineered and LB1 was employed to monitor the intracellular luteolin, as LB2 might be interfered by the eriodictyol when detecting low luteolin concentration (Figure 4B). To enable luteolin production, plasmid pE6K-FNS was constructed, which encodes the Flavonoid Synthase (FNS) enzyme responsible for converting eriodictyol to luteolin. The FNS expression was under the control of an IPTG-inducible promoter. The LB1 and pE6K-FNS were then co-transformed into *E. coli*. When eriodictyol and IPTG were added into the cell culture, the FNS enzyme would be expressed, leading to the conversion of eriodictyol to luteolin. The intracellularly produced luteolin would then activate LB1, enabling the translation of GFP and generating the fluorescence signal, as illustrated in Figure 5A.

[00137] To study the capability of LB1 in detecting various concentration of intracellular luteolin, IPTG concentration gradient was set at 0, 50, 100, 200, 300 μM to vary FNS expression levels, while substrate eriodictyol concentration was fixed at 105 μM due to the solubility limitation. The cell growth and GFP levels were continuously monitored over 24 hours at 30°C, in which temperature the enzyme would have better stability. Figure 5B showed GFP/OD increased with higher IPTG concentration, with fold changes of 3.1, 5.4, 7.4, and 9.6 at 50, 100, 200, 300 μM IPTG, respectively. At the endpoint of the cell culture, HPLC was used to confirm the luteolin concentrations produced by *E. coli* (Figure 5C, Figure 20). HPLC measurement results showed that the luteolin concentrations produced by *E. coli* increased with higher IPTG concentration (Table 6). The HPLC analysis confirmed that BL1 exhibited significant responsiveness to 2.25 μM luteolin that produced by *E. coli*, demonstrating a low intracellular detection limit. A strong linear correlation ($R^2 = 0.93$) was observed between GFP/OD signals and luteolin concentrations measured by HPLC (Figure 5D), validating the biosensor's accuracy in detecting the intracellular luteolin. Interestingly, although the intracellularly produced luteolin concentration was lower than the externally supplied concentrations tested previously (Figure 3), the biosensor showed stronger GFP expression. This enhanced response is likely due to better accessibility of intracellular luteolin to the biosensor. Despite that the biosensor might also respond to eriodictyol, as seen in Figure 4, the biosensor's readouts reliably indicated the luteolin concentrations even in the presence of eriodictyol. These results suggest that the biosensor can effectively detect intracellular luteolin production, enabling real-time monitoring of the biosynthesis process, as well as other flavonoid-related *in vivo* applications.

[00138] **Table 6. Direct comparative data between biosensor and HPLC analysis results, in the in vivo luteolin sensing experiment.**

IPTG conc. (μM)	GFP/OD (AU) signal generated biosensor	Luteolin conc. (μM) measured by HPLC
0	2079.23	0
50	6348.56	2.2484
100	11239.99	6.069
200	15337.28	11.9616
300	20006.11	22.2278

[00139] ***2.6 Performance of the biosensor at various temperatures***

[00140] Testing a biosensor at different temperatures is crucial for microbial engineering, since cell growth, enzymatic activity, protein stability, and biosensor regulatory elements may vary with temperature. To this end, the LB2 was tested at 18, 25, 30, 37, and 40 °C, with luteolin concentrations of 0, 1.75, 17.5, 35, 70, and 105 μM (Figure 21). The sensor showed highest GFP expression at 37 °C in response to different luteolin concentrations (Figure 21D). The GFP expression at 18 and 25 °C (Figure 21A-B) exhibited a lower level as compared to 30 and 37 °C (Figure 21C-D). Regardless, the LB2's output increased when increasing luteolin concentration in the temperature 18 - 37 °C, demonstrating its robustness across a broad temperature range. Although the sensor's output showed an increasing trend from luteolin concentrations of 0 - 35 μM at 40 °C, however, a weaker correlation between sensor output and luteolin concentration at 70 and 105 μM were observed (Figure 21E).

[00141] **Example 3 – Conclusions**

[00142] In this work, two novel luteolin-responsive riboswitch biosensors that demonstrate good sensitivity and specificity were developed. These biosensors are capable of both *in vitro* and *in vivo* detection of luteolin. They showed significant responses to luteolin within 50-60 minutes and exhibited operational range of 1.75 - 105 μM, with a fold change of up to 9.2. Notably, they exhibited responsiveness at luteolin concentrations as low as 1.75 μM, showcasing a low detection limit. Moreover, the biosensors demonstrated robust performance under various conditions, including different temperatures, culture medium, and host strains, confirming their stability and reproducibility. Importantly, the present biosensors effectively detected intracellular luteolin produced by *E. coli*, achieving up to 9.6-fold change and displaying consistent

results with HPLC analysis. Additionally, these biosensors also demonstrated applications for detecting other flavonoids (apigenin), further extending their utility.

[00143] Unlike previous riboswitch development studies rely on RNA oligos for SELEX [34, 51], the present approach utilized DNA, which offers greater stability and simplified operational procedures. By integrating DNA-based SELEX with replica methods, a streamlined strategy for riboswitch development was established. This work developed two biosensors with different sensitivity, dynamic range, specificity, and fold activation. Having the two biosensors expands their applications, allowing utility in different flavonoid concentrations, types, and pathways. Overall, these biosensors represent valuable tools for flavonoid-related applications in flavonoid analytics and *in vivo* sensing, with potential uses in, high-throughput screening [59], real-time monitoring [60], metabolic engineering [14], and biosynthesis pathways regulation [16].

[00144] The disclosed biosensors addressed an important limitation in the sensing of luteolin and other naringenin derived flavonoids and represented a significant step towards achieving efficient and sustainable production of luteolin and other naringenin derived flavonoids. To the best of the inventors' knowledge, there is no riboswitch of luteolin and other naringenin derived flavonoids available in the market or reported in the literature.

[00145] Commercial applications for these luteolin biosensors include:

i) Using this biosensor to develop detection kits of luteolin and other naringenin derived flavonoids for point of use.

ii) Using the biosensor for ultra-high throughput screening of the luteolin- and other naringenin derived flavonoids- producing enzyme/strains. This will greatly reduce the labour and time requirements, accelerating the biomanufacturing of luteolin and other naringenin derived flavonoids for market.

iii) Dynamic regulation of biosynthesis pathways to control and optimize bioproduction of luteolin and other naringenin derived flavonoids.

iv) Food quality control, for example, determination of luteolin and other naringenin derived flavonoids in Chrysanthemum tea.

v) Bioprocess optimization for scaling up, monitoring of luteolin and other naringenin derived flavonoids produced by microbes in bioreactor.

[00146] The biosensor as disclosed herein offers several significant advantages:

[00147] First, the novel biosensor enables the detection of luteolin and other naringenin derived flavonoids both *in vitro and in vivo*. It is able to measure the concentration of luteolin and other naringenin derived flavonoids *in vivo* right after it is produced inside the cell, providing a more accurate measurement of the biosynthesis of luteolin and other naringenin derived flavonoids, unlike conventional sensors (e.g., HPLC, electrochemical sensors) that can only measure the target concentration *in vitro* (outside the cell).

[00148] Second, the disclosed biosensor is a whole cell-based living biosensor, which allows single cell real-time monitoring the concentration of luteolin and other naringenin derived flavonoids at single cell level, avoid the need to do sample preparation and running HPLC which is tedious, time consumption and limited in throughput.

[00149] Third, the biosensor as disclosed herein can be easily fabricated at low cost (about SGD 0.0035 per sensing reaction), and it is possible to grow the cell to expand the biosensor for larger quantity measurement.

[00150] Forth, the biosensor as disclosed herein has a wide dynamic sensing range of 5-120 μM, that is sufficient to cover the working concentration for detection of luteolin and other naringenin derived flavonoids, and relevant strain/enzyme screening.

[00151] Finally, the biosensor as disclosed herein enables ultra-high throughput screening for strain/enzymes.

[00152] **Industrial Applicability**

[00153] It will be apparent that various other modifications and adaptations of the invention will be apparent to the person skilled in the art after reading the foregoing disclosure without departing from the spirit and scope of the invention and it is intended that all such modifications and adaptations come within the scope of the appended claims.

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Claims

1. An aptamer capable of binding to luteolin and other naringenin derived flavonoids, wherein the aptamer is: **an RNA aptamer** having a nucleotide sequence selected from the group consisting of: GUCCNNNNUCAGUCNNNNGACUGAGGAU (SEQ ID NO: 1), and
   AUGCUGGNNNNNCUAGUAUNNNNACAUNNNNAUGU (SEQ ID NO: 2); or
   a DNA aptamer having a nucleotide sequence selected from the group consisting of: GTCCNNNNTCAGTCNNNNGACTGAGGAT (SEQ ID NO: 3), and
   ATGCTGGNNNNNCTAGTAT (SEQ ID NO: 4);
   wherein for SEQ ID NO: 1 and 2, N is one nucleotide selected from the group consisting of A, G, C and U, and
   wherein for SEQ ID NO: 3 and 4, N is one nucleotide selected from the group consisting of A, G, C and T.
2. The aptamer of claim 1, wherein the other naringenin derived flavonoids is selected from the group consisting of apigenin and eriodictyol.
3. The aptamer of claim 2, wherein the aptamer having a nucleotide sequence of SEQ ID NO: 1 or SEQ ID NO: 3 is capable of binding to luteolin or eriodictyol, and wherein the aptamer having a nucleotide sequence of SEQ ID NO: 2 or SEQ ID NO: 4 is capable of binding to luteolin or apigenin.
4. A riboswitch comprising the RNA aptamer of any one of claims 1-3, a ribosomal binding site (RBS) and a reporter; wherein the RNA aptamer comprises the nucleotide sequence of SEQ ID NO: 1 or SEQ ID NO: 2.
5. The riboswitch of claim 4, wherein the RBS is selected from the group consisting of *E.coli* RBS having a consensus sequence of AGGA, a default RBS having a sequence of tttaagaaggagatatacat, and RBS34 having a sequence of tctagagaaagaggagaaatactag.
6. The riboswitch of claim 4, wherein the reporter is selected from the group consisting of fluorescence protein, fluorophore, colour dye, luminophore, and luciferase.
7. The riboswitch of claim 6, wherein the fluorescence protein is selected from the group consisting of a green fluorescence protein selected from the group consisting of EGFP, Emerald, Superfolder GFP, Azami Green, mWasabi, TagGFP, TurboGFP, AcGFP, ZsGreen and T-Sapphire; a blue fluoresce protein selected from the group consisting of EBFP, EBFP2, Azurite and mTagBFP; a cyan fluoresce protein selected from the group consisting of ECFP, mECFP, Cerulean, mTurquoise, CyPet,

AmCyan1, Midori-Ishi Cyan, TagCFP and mTFP1 (Teal); a yellow fluorescence protein selected from the group consisting of EYFP, Topaz, Venus, mCitrine, YPet, TagYFP, PhiYFP, ZsYellow1 and mBanana; an orange fluorescence protein selected from the group consisting of Kusabira Orange, Kusabira Orange2, mOrange, mOrange2, dTomato, dTomato-Tandem, TagRFP, TagRFP-T, DsRed, DsRed2, DsRed-Express (T1), DsRed-Monomer and mTangerine; and a red fluorescence protein selected from the group consisting of mRuby, mApple, mStrawberry, AsRed2, mRFP1, JRed, mCherry, HcRed1, mRaspberry, dKeima-Tandem, HcRed-Tandem, mPlum, and AQ143.

8. A DNA expression cassette for the regulation of the expression of the reporter of any one of claims 4-7, comprising a promoter, a DNA aptamer of any one of claims 1-3, a DNA sequence encoding the RBS and a DNA sequence encoding the reporter of the riboswitch of any one of claims 4-7.
9. The DNA expression cassette of claim 8, wherein the promoter is selected from the group consisting of a *E. coli* σ^{70}, σ^{19}, σ^{24}, σ^{28}, σ^{32}, σ^{38}, and σ^{54} promoter.
10. A composition for detecting luteolin and other naringenin derived flavonoids, comprising the riboswitch of any one of claims 4-7, and a translation mixture.
11. A composition for detecting luteolin and other naringenin derived flavonoids, comprising the DNA expression cassette of claim 8 or 9, a transcription mixture and a translation mixture.
12. The composition of claim 10 or 11, wherein the translation mixture is a *E. coli* cell-free translation mixture.
13. The composition of claim 11, wherein the transcription mixture is *E. coli* cell-free transcription mixture.
14. A microbe containing the riboswitch of any one of claims 4-7 or the DNA expression cassette of claim 8 or 9.
15. The microbe of claim 14, wherein the microbe is a prokaryote selected from the group consisting of *E. coli*, *B. subtilis*, Pseudomonas, Lactobacillus, Spirilla, Vibrio, Salmonella, Acetobacter, Cyanobacteria, Spirochetes, Chlamydias, Caulobacter, Mycoplasma, Aliivibrio, Bacteroides, Synechocystis, Azotobacter, Streptomyces, Thermococcus, Sulfolobus, Streptococcus, Methanococcus, Halobacterium, other proteobacteria and a gram-positive bacterium.

16. A method of detecting luteolin and other naringenin derived flavonoids produced in a microbe, comprising (a) introducing the riboswitch of any one of claims 4-7 or the DNA expression cassette of claim 8 or 9 into the microbe, wherein when the microbe produces the luteolin and the other naringenin derived flavonoids in an effective amount, the luteolin and the other naringenin derived flavonoids specifically bind the aptamer to induce a conformational change in the riboswitch that allows translation of the reporter into a reporter protein; and

(b) detecting the reporter protein, wherein the presence of the reporter protein is indicative of detection of the luteolin and other naringenin derived flavonoids.

17. A method of detecting luteolin and other naringenin derived flavonoids produced by a microbe, comprising

(a) incubating the microbe with the composition of claim 10 or the composition of claim 11 in a medium, wherein when the microbe produces and releases the luteolin and the other naringenin derived flavonoids in an effective amount into the medium, the luteolin and the other naringenin derived flavonoids specifically bind the aptamer of the riboswitch to induce a conformational change in the riboswitch that allows translation of the reporter into a reporter protein; and

(b) detecting the reporter protein, wherein the presence of the reporter protein is indicative of detection of the luteolin and other naringenin derived flavonoids.

18. A method of detecting luteolin and other naringenin derived flavonoids produced by a first microbe, comprising

(a) incubating the first microbe and the microbe of claim 14 or 15 in a medium; wherein when the first microbe produces and releases the luteolin and the other naringenin derived flavonoids in an effective amount into the medium, the luteolin and the other naringenin derived flavonoids specifically bind the aptamer of the riboswitch produced by the microbe of claim 14 or 15 to induce a conformational change in the riboswitch that allows translation of the reporter into a reporter protein; and

(b) detecting the reporter protein, wherein the presence of the reporter protein is indicative of detection of the luteolin and the other naringenin derived flavonoids.

19. The method of any one of claims 16-18, wherein the microbe is a prokaryote selected from the group consisting of *E. coli*, *B. subtilis*, Pseudomonas, Lactobacillus, Spirilla, Vibrio, Salmonella, Acetobacter, Cyanobacteria, Spirochetes, Chlamydias,

Caulobacter, Mycoplasma, Aliivibrio, Bacteroides, Synechocystis, Azotobacter, Streptomyces, Thermococcus, Sulfolobus, Streptococcus, Methanococcus, Halobacterium, other proteobacteria and a gram-positive bacterium.

20. The method of any one of claims 16-19, wherein the other naringenin derived flavonoids are selected from the group consisting of apigenin and eriodictyol.
21. The method of claim 20, wherein the luteolin or the other naringenin derived flavonoids have a concentration of at least 1 μM, at least 1.75 μM, at least 2 μM, at least 2.25 μM, at least 5 μM, at least 7.5 μM, at least 10 μM, at least 15 μM, at least 17.5 μM, at least 35 μM, at least 70 μM, or at least 105 μM.
22. The method of any one of claims 16-21, wherein the reporter protein is detected by fluorescent detection, colorimetric detection, bioluminescence detection, spectrophotometry, and DNA microarray.
23. The method of any one of claims 16-22, wherein the level of the reporter protein translated is at least about 1.24-fold higher when the luteolin and the other naringenin derived flavonoid is present than the level when the luteolin and the other naringenin derived flavonoid is absent.
24. The method of any one of claims 16-23, wherein the method is performed at a temperature of 18-37 °C.