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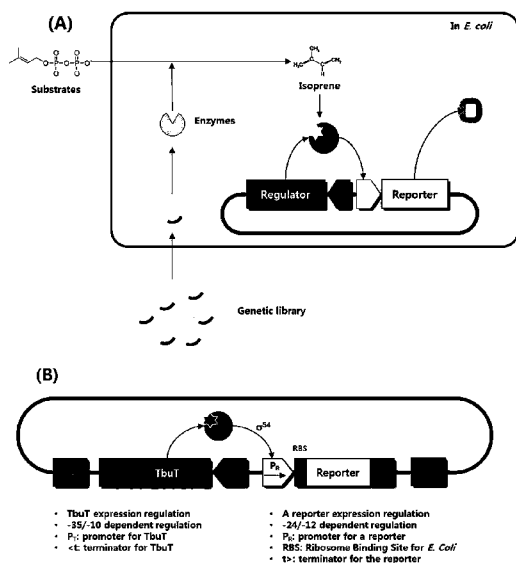
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(54) **Title:** METHOD FOR SCREENING AND QUANTIFYING ISOPRENE BIOSYNTHESIS ENZYME ACTIVITY



(57) **Abstract:** The present invention is a novel technology of performing high-throughput screening of various enzymatic activities with high sensitivity using artificial genetic circuits. More specifically, the invention is a novel screening and quantifying methods of isoprene biosynthesis enzymes activity using an artificial genetic circuit capable of sensing isoprene. The artificial genetic circuit (IspGESS) is comprising a gene encoding a isoprene-sensing transcriptional regulator which recognizes isoprene, at least one reporter gene such fluorescence protein-encoding genes, a isoprene-sensing transcriptional regulator binding region and promoters for genes encoding isoprene-sensing transcriptional regulators and reporter proteins. IspGESS detects isoprene liberated from many enzymatic reactions and allows to measure the activity of reporter genes, such as fluorescent reporter genes and antibiotic resistance genes. This system is shown that it is a widely applicable tool for high throughput and quantitative screening of isoprene biosynthesis enzymes and MEP/MVA pathway enzymes. Thus, the invention can be advantageously used in the protein engineering technology for enzyme modification. Particularly, it can quantitatively investigate enzymatic activity, and thus can be applied to molecular evolution technology.



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Description

Title of Invention: Method for Screening and Quantifying Isoprene Biosynthesis Enzyme Activity

Technical Field

- [1] The present invention relates to a novel genetic method of detecting and quantifying target enzyme activity. More specifically, the invention relates to a novel method of screening isoprene biosynthesis enzyme activity using an artificial genetic circuit capable of sensing isoprene. The method can be applied for the improvement of isoprene biosynthesis enzymes and pathways in bacterial system.

[2]

Background Art

- [3] Biocatalysts are recognized as one of key component for “sustainable chemistry development”, such as the biological syntheses of biopolymers, bioenergy, industrial chemicals etc, and various efforts have been made to obtain better enzymes having new chemical reactivity, specificity, and stability. However, mining of DNA sequence databases have problems like limited information on new catalytic activities and screenings using classical methods such as instrumental analysis of enzyme products have been suffered by low throughput and high cost of assay methods. In addition, although some industrial enzymes (e.g., amylase, lipase, protease, etc.) have been screened by the examination of cell growth or halo formation on solid media but the correlation between the phenotype and the enzyme activity are hardly quantitative.
- [4] Thus, there has been demands for the development of high-throughput and quantitative methods to detect the activities of industrially important enzymes. A quantitative screening technology will help to identify new biocatalysts from microbial genomes or metagenomes that have been emerged as important resources in modern biotechnology. Furthermore, the technology can be applied to enable the effective engagements of directed evolution technology to acquire chemical reactivity, specificity, and stability of enzymes from existing genes.
- [5] Among enzymes in biosynthesis pathways, isoprene synthase (IspS) catalyzes a key step that can produce isoprene which is used as a raw material for automobile tires and medical supplies. To date, the quantification of isoprene produced by the activity of IspS has relied mainly on the use of gas chromatography (GC). In the method, isoprene synthesized in complicated fermentation facilities such as large-scale bioreactor was monitored after the emission gas was captured from the headspace of fermentation equipment. Therefore, the GC analysis is not an effective method for the rapid screening or directed evolution that requires handling large sizes of genetic libraries.

The sampling and GC analyses of isoprene gas is too time consuming, costly, and laborious to be used in search of new enzymes from a large size library. Thus, there is a large demand for a method that can measure the activities of enzymes in a simpler and more rapid and cost-effective manner.

[6] Accordingly, the present inventors have conducted studies on a high throughput and quantitative method capable of detecting isoprene compounds. The inventors have paid attention to the characteristics of regulatory proteins which detect isoprene in bacterial cells and designed an artificial genetic circuit recognizing isoprene biosynthetic products. As a result, the inventors found that the genetic circuit enables quantitative measurements of the IspS activity in bacterial systems. The present inventors have confirmed that invented technique can be efficiently and generally used for detecting improved enzyme activities from an IspS mutant library using a high-throughput flow cytometry (million/day). The inventors understand the present invention can be further extended to evaluate the effects of any enzymes' regulators, or metabolic pathways that affecting to the isoprene synthesis, not only the case of IspS, but also other related pathway genes.

[7] The above information disclosed in this Background section is only for enhancement of understanding of the background of the invention and therefore it may contain information that does not form the prior art that is already known in this country to a person of ordinary skill in the art.

[8]

[9] Disclosure of Invention

[10] It is an object of the present invention to provide a method of detecting and screening isoprene biosynthesis enzyme activity using an artificial genetic circuit which recognizes isoprene.

[11] Another object of the present invention is to provide a method of quantifying isoprene biosynthesis enzyme activity using an artificial genetic circuit which recognizes isoprene.

[12]

[13] To achieve the above objects, the present invention provides a method of screening (detecting) one or more isoprene biosynthesis enzymes, activities using an artificial genetic circuit, the method comprising the steps of:

[14] (a) providing an artificial genetic circuit for detecting isoprene, the artificial genetic circuit comprising (i) a gene encoding an isoprene-sensing transcriptional regulator which recognizes isoprene, (ii) at least one reporter gene selected from the group consisting of fluorescence protein-encoding genes and antibiotic resistance genes, and (iii) a gene expression regulatory region consisting of a promoter regulating the expression of the isoprene-sensing transcriptional regulator, a region to which the

isoprene-sensing transcriptional regulator binds to induce the expression of a downstream reporter gene, and a promoter regulating the expression of the reporter gene;

[15] (b) providing a clone or gene library containing one or more of a gene encoding an isoprene biosynthesis enzyme;

[16] (c) introducing the clone or gene library and the artificial gene circuit for detecting isoprene into host microorganisms to prepare recombinant microorganisms;

[17] (d) treating the recombinant microorganisms with a compound capable of liberating isoprene by an enzymatic reaction; and

[18] (e) detecting the activity of the reporter protein whose expression is induced by sensing isoprene liberated by the enzymatic reaction.

[19] The present invention also provides a method of screening one or more isoprene biosynthesis enzymes' activities using an artificial genetic circuit, the method comprising the steps of:

[20] (a) providing microorganisms containing in their chromosomal DNA or cytoplasm an artificial genetic circuit for detecting isoprene, the artificial genetic circuit comprising (i) a gene encoding an isoprene-sensing transcriptional regulator which recognizes isoprene, (ii) at least one reporter gene selected from the group consisting of fluorescence protein-encoding genes and antibiotic resistance genes, and (iii) a gene expression regulatory region consisting of a promoter regulating the expression of the isoprene-sensing transcriptional regulator, a region to which the isoprene-sensing transcriptional regulator binds to induce the expression of a downstream reporter gene, and a promoter regulating the expression of the reporter gene;

[21] (b) providing a clone or gene library containing one or more of a gene encoding an isoprene biosynthesis enzyme;

[22] (c) introducing the clone or gene library into the microorganisms containing the artificial gene circuit for detecting isoprene to prepare recombinant microorganisms;

[23] (d) treating the recombinant microorganisms with a compound capable of liberating isoprene by an enzymatic reaction; and

[24] (e) detecting the activity of the reporter protein whose expression is induced by sensing isoprene liberated by the enzymatic reaction.

[25] The present invention also provides a method of quantifying one or more isoprene biosynthesis enzymes activities using an artificial genetic circuit, the method comprising the steps of:

[26] (a) providing an artificial genetic circuit for detecting isoprene, the artificial genetic circuit comprising (i) a gene encoding an isoprene-sensing transcriptional regulator which recognizes isoprene, (ii) at least one reporter gene selected from the group consisting of fluorescence protein-encoding genes and antibiotic resistance genes, and

- (iii) a gene expression regulatory region consisting of a promoter regulating the expression of the isoprene-sensing transcriptional regulator, a region to which the isoprene-sensing transcriptional regulator binds to induce the expression of a downstream reporter gene, and a promoter regulating the expression of the reporter gene;
- [27] (b) providing a clone or gene library containing one or more of a gene encoding an isoprene biosynthesis enzyme;
- [28] (c) introducing the clone or gene library and the artificial gene circuit for detecting isoprene into host microorganisms to prepare recombinant microorganisms;
- [29] (d) treating the recombinant microorganisms with a compound capable of liberating isoprene by an enzymatic reaction; and
- [30] (e) quantifying the activity of the reporter protein whose expression is induced by sensing isoprene liberated by the enzymatic reaction.
- [31] The present invention also provides a method of quantifying one or more isoprene biosynthesis enzymes using an artificial genetic circuit, the method comprising the steps of:
- [32] (a) providing microorganisms containing in their chromosomal DNA or cytoplasm an artificial genetic circuit for detecting isoprene, the artificial genetic circuit comprising (i) a gene encoding a isoprene-sensing transcriptional regulator which recognizes isoprene, (ii) at least one reporter gene selected from the group consisting of fluorescence protein-encoding genes and antibiotic resistance genes, and (iii) a gene expression regulatory region consisting of a promoter regulating the expression of the isoprene-sensing transcriptional regulator, a region to which the isoprene-sensing transcriptional regulator binds to induce the expression of a downstream reporter gene, and a promoter regulating the expression of the reporter gene;
- [33] (b) providing a clone or gene library containing one or more of a gene encoding an isoprene biosynthesis enzyme;
- [34] (c) introducing the clone or gene library into the microorganisms containing the artificial gene circuit for detecting isoprene to prepare recombinant microorganisms;
- [35] (d) treating the recombinant microorganisms with a compound capable of liberating isoprene by an enzymatic reaction; and
- [36] (e) quantifying the activity of the reporter protein whose expression is induced by sensing isoprene liberated by the enzymatic reaction.
- [37] The present invention also provides an artificial genetic circuit for detecting isoprene, the artificial genetic circuit comprising (i) a gene encoding an isoprene-sensing transcriptional regulator which recognizes isoprene, (ii) at least one reporter gene selected from the group consisting of fluorescence protein-encoding genes and antibiotic resistance genes, and (iii) a gene expression regulatory region consisting of a promoter

regulating the expression of the isoprene-sensing transcriptional regulator, a region to which the isoprene-sensing transcriptional regulator binds to induce the expression of a downstream reporter gene, and a promoter regulating the expression of the reporter gene.

[38] The present invention also provides a recombinant microorganism containing said artificial genetic circuit.

[39] The present invention also provides a method of quantifying isoprene using said artificial genetic circuit, the method comprising the steps of:

[40] (a) introducing the artificial gene circuit of claim 19 into host microorganisms to be measured; and

[41] (b) quantifying isoprene by measuring the activity of the reporter protein whose expression is induced by sensing isoprene.

[42] The present invention also provides a gene encoding IspSm2 of SEQ ID NO: 8.

[43]

Brief Description of Drawings

[44] FIG. 1(A) schematically shows a principle (GFP-based Enzyme Screening System for isoprene (IspGESS)) of screening isoprene biosynthesis enzyme activity using an artificial genetic circuit according to the present invention, and FIG. 1(B) schematically shows an IspGESS vector (pIspGESS) and also shows an enlarged structure of the gene expression regulatory region of the IspGESS vector. In FIG. 1, Regulator: transcriptional regulation factor; PT: promoter regulating the expression of an isoprene-sensing transcriptional regulator; and PR: promoter regulating the expression of a reporter.

[45] FIG. 2 shows a process of constructing pIspGESS. Specifically, PCR products of *tbuT* gene and P_{tbuA} are derived from *R. pickettii*. PCR product of P_{hcc} is a highly constitutive promoter for expression of reporter protein. The three PCR products are combined by overlap PCR and cloned into the pGESS (USP 13/376,783).

[46] FIG. 3 shows the verification of IspGESS by measuring quantitative responses to various compounds using fluorescence-activated cell sorting (FACS). (A): the measurement of the quantitative response of IspGESS to isoprene; (B): the measurement of the quantitative response of IspGESS to toluene; (C) the measurement of the quantitative response of IspGESS to phenol; and (D) the measurement of the quantitative response of IspGESS to benzene.

[47] FIG. 4 shows that IspGESS can detect the isoprene synthase (IspSm1) activity using fluorescence-activated cell sorting (FACS). IspGESS with IspSm1 (FIG. 4 right) shows higher fluorescence intensity than that without IspSm1 (FIG. 4. left).

[48] FIG. 5 shows a process of performing high-throughput screening of IspS from a

mutant IspSm1 library using the IspGESS.

- [49] FIG. 6 shows the verification of activity of positive hit (IspSm2) selected from the IspSm1 random mutant library. Three columns represent *E. coli* BL21(DE3) strains containing IspGESS only (left), IspGESS with IspSm1 (center), and IspGESS with IspSm2 (right), respectively. (A) the results of analyzing the fluorescence image of a colony on solid medium; and (B) the results of analyzing the intensity of fluorescence in liquid medium by fluorescence-activated cell sorting (FACS).
- [50] FIG. 7 shows the amino acid sequence of the IspSm2 having increased activity contains a lysine-to-arginine mutation at position 35 from the amino acid sequence of IspSm1.
- [51] FIG. 8 shows the verification of activity of IspSm1 and IspSm2 in pPROLar vector by fluorescence-activated cell sorting (FACS). Three columns represent *E. coli* DH5a strains containing IspGESS only (left), IspGESS with pPROLar-IspSm1 (center), and IspGESS with pPROLar-IspSm2 (right), respectively.
- [52] FIG. 9 shows the results of t-test for IspSm1 and IspSm2 activities with respect to no IspS control.
- [53] FIG. 10 shows the verification of IspGESSHCE and IspGESSTRC by measuring the responses to isoprene using FACS.
- [54] FIG. 11 (A) schematically shows an IspGESSTRC_sfGFP and also shows an enlarged structure of the gene expression regulatory region of the IspGESSTRC_sfGFP vector, FIG. 11 (B) shows Fluorescence intensity of IspGESSTRC_sfGFP by measuring the responses to isoprene with various range (0~2000 μ M) using FACS. And FIG. 11 (C) shows the verification of IspGESSTRC and IspGESSTRC_sfGFP by measuring the responses to isoprene with various range (0~2000 μ M) using FACS.
- [55] FIG. 12 schematically shows a T7IspGESS_{TRC}_sfGFP and also shows an enlarged structure of the gene expression regulatory region of the T7IspGESS_{TRC}_sfGFP vector.
- [56] FIG. 13 (A) shows the verification of IspGESS_{TRC}, IspGESS_{HCE}, T7IspGESS_{TRC} and T7IspGESS_{HCE} by measuring the responses to isoprene using fluorometer and FIG. 13 (B) shows verification of T7IspGESSTRC by measuring the responses to isoprene with various range using fluorometer.
- [57] FIG. 14 shows the verification of IspGESS_{TRC}, T7IspGESS_{TRC} and T7IspGESS_{TRC}_sfGFP by measuring the responses to isoprene using fluorometer.
- [58] FIG. 15 shows the verification of T7IspGESS_{TRC}_sfGFP, T7IspGESS_{J23105}_sfGFP and T7IspGESS_{J23114}_sfGFP by measuring the responses to isoprene using fluorometer.
- [59]
- [60] Best Mode for Carrying Out The Invention
- [61] Unless defined otherwise, all technical and scientific terms used herein have the same

meaning as commonly understood by one of ordinary skill in the art to which the invention pertains. Generally, the nomenclature used herein is well known and commonly employed in the art.

- [62] For severe climate change and petroleum resources depletion leading to strict environmental regulation and upside price risk of oil refinery products, it is critical concerns to replace oil-based chemical feedstock with biomass-based eco-friendly materials emitting low green-house gas. Isoprene is a colorless, highly volatile compound having a melting point of -145.95°C , a boiling point of 34.067°C and a density of 0.68 g/cm^3 . It is obtained as a byproduct of cracking of naphtha/oil and is mainly used for the production of synthetic rubber (cis-1,4-polyisoprene) which is used as a main raw material for tires in the automobile industry. In addition, it is used as a raw material for paint, the major component of medical devices, and the like. As an alternative source of isoprene derived from petroleum, bioisoprene can be produced by means of microbial synthesis of isoprene by fermentation and collected from the gas phase of the fermentor, eliminating the need for distillation. The world market size of bioisoprene is over 1 ~ 2 billion dollars in 2007 and the market size of 2013 is predicted over 12 billion dollars.
- [63] Isoprene is biosynthesized from the same basic units, isopentenyl diphosphate (pyrophosphate IPP), and its isomer dimethylallyl diphosphate (DMAPP), which are synthesized from two different pathways including methylerythritol 4-phosphate (MEP) pathway and mevalonate (MVA) pathway. MVA pathway mainly exists in eukaryotes, archaeobacteria, and cytosols of higher plants, while the MEP pathway is used by many eubacteria, green algae, and chloroplasts of higher plant. MVA pathway has been studied extensively for producing isoprene. The introduction of heterologous MVA pathway genes into *E. coli* has been reported to improve the productivity of carotenoids or sesquiterpenes that are synthesized from DMAPP (Yang J *et al.* (2012) PLoS ONE 7(4): e33509).
- [64] Isoprene synthase (EC 4.2.3.27) is an enzyme that catalyzes the chemical reaction producing isoprene and diphosphate from dimethylallyl diphosphate (DMAPP). Isoprene is highly volatile, and thus is very difficult to analyze quantitatively, and gas chromatography analysis which is generally performed requires a significant amount of isoprene.
- [65] TbuT is one of NtrC family of transcriptional activator that regulates toluene-3-monooxygenase operon (tbuA1UBVA2C).
- [66] The NtrC family regulator consists of a combination of a domain (domain A) recognizing aromatic compounds such as toluene or phenol, a domain (domain C) having ATPase activity, and a domain (domain D) functioning to bind to DNA. Thus, when there is no aromatic compound, domain A inhibits transcription, but when an aromatic

compound binds to domain A, domains C and D are activated for transcription of downstream structural genes. In recent years, a study on the use of domain A to detect new substances and a study on the modification of specificity by domain A were reported (Pavel *et al.*, (1994) J. Bacteriol. 176(4): 7550-7557).

- [67] Typical NtrC family transcriptional activators which are known in the art include XylR, TbuT, TbmR, PcuR, MopR, TouR, PhlR and DmpR the like, and among them, the most well-known are XylR, which is involved in the metabolism of toluene and xylene in *Pseudomonas putida* (Ramos & Marques, (1997), and *Annu.Rev.Microbiol.* 51:341-372), and TbuT which is involved in the toluene degradation metabolism (Armando M. Byrne et. & RONALD H. OLSEN, (1996), and J. of Bacteriol., 6327?6337). These Ntrc family activators regulate a multifunctional operon in which a plurality of genes are expressed together, and these genes are expressed by regulation of σ^{54} -dependent transcription.
- [68] It is known that σ^{54} -dependent transcriptional regulator TbuT senses toluene and benzene, and activates expression of the *tbuA1UBVA2C* operon by binding to a P_{tbuA} promoter. Expression of *tbuT* and that of the *tbuA1UBVA2C* operon are linked by readthrough transcription of *tbuT* from the toluene-3-monooxygenase promoter. Transcription of *tbuT* is low when the toluene-3-monooxygenase operon is uninduced and high when the expression of *tbuA1UBVA2C* is induced by toluene. Thus, the toluene-3-monooxygenase promoter drives the cascade expression of both the toluene-3-monooxygenase operon and *tbuT*, resulting in a positive feedback circuit.
- [69] It is generally known that TbuT is activated by aromatic compounds including toluene, benzene, naphthalene, catechol and chlorobenzene, but it responded slightly towards non-aromatic compounds.
- [70] The investigating method for isoprene biosynthesis enzyme activity according to the present invention requires constructing an artificial genetic circuit for sensing of isoprene released from a substrate. For this, in the present invention, GESS obtained in previous studies (USP 13/376,783) was redesigned, thereby constructing the novel genetic circuit, IspGESS.
- [71] GESS (GFP-based enzyme screening system) is a technology of performing high-throughput screening of various enzymatic activities with high sensitivity using artificial genetic circuits. DmpR based GESS detects phenols liberated from many enzymatic reactions and allows to measure the activity of reporter genes, such as fluorescent reporter genes and antibiotic resistance genes. This system is shown that it is a widely applicable tool for high throughput and quantitative screening of diverse enzymes such as phosphatase, lipase, oxydoreductase, cellulase and so on.
- [72] Based on the GESS, a genetic circuit with a *R. pickettii*-derived TbuT expression regulator was constructed by a cloning process for replacing the *dmpR* of the GESS

plasmid.

- [73] In one aspect, the present invention is directed to a method of screening one or more isoprene biosynthesis enzymes' activities using an artificial genetic circuit, the method comprising the steps of: (a) providing an artificial genetic circuit for detecting isoprene, the artificial genetic circuit comprising (i) a gene encoding an isoprene-sensing transcriptional regulator which recognizes isoprene, (ii) at least one reporter gene selected from the group consisting of fluorescence protein-encoding genes and antibiotic resistance genes, and (iii) a gene expression regulatory region consisting of a promoter regulating the expression of the isoprene-sensing transcriptional regulator, a region to which the isoprene-sensing transcriptional regulator binds to induce the expression of a downstream reporter gene, and a promoter regulating the expression of the reporter gene; (b) providing a clone or gene library containing one or more of a gene encoding an isoprene biosynthesis enzyme; (c) introducing the clone or gene library and the artificial gene circuit for detecting isoprene into host microorganisms to prepare recombinant microorganisms; (d) treating the recombinant microorganisms with a compound capable of liberating isoprene by an enzymatic reaction; and (e) detecting the activity of the reporter protein whose expression is induced by sensing isoprene liberated by the enzymatic reaction.
- [74] According to the present invention, the technology (GESS: GFP-based enzyme screening system) of sensing various enzymatic activities using an artificial genetic circuit with high sensitivity in a simple manner is applied to investigate isoprene biosynthesis enzyme activity. The present invention provides a method which enables to investigate efficiently isoprene biosynthesis enzyme activity from large libraries, which was not easy to investigate by conventional screening methods. The method of the present invention also has an advantage in that isoprene biosynthesis enzyme activity can be quantitatively measured.
- [75] In another aspect, the present invention also provides a method of screening one or more isoprene biosynthesis enzymes' activities using an artificial genetic circuit, the method comprising the steps of: (a) providing microorganisms containing in their chromosomal DNA or cytoplasm an artificial genetic circuit for detecting isoprene, the artificial genetic circuit comprising (i) a gene encoding an isoprene-sensing transcriptional regulator which recognizes isoprene, (ii) at least one reporter gene selected from the group consisting of fluorescence protein-encoding genes and antibiotic resistance genes, and (iii) a gene expression regulatory region consisting of a promoter regulating the expression of the isoprene-sensing transcriptional regulator, a region to which the isoprene-sensing transcriptional regulator binds to induce the expression of a downstream reporter gene, and a promoter regulating the expression of the reporter gene; (b) providing a clone or gene library containing one or more of a gene encoding

an isoprene biosynthesis enzyme; (c) introducing the clone or gene library into the microorganisms containing the artificial gene circuit for detecting isoprene to prepare recombinant microorganisms; (d) treating the recombinant microorganisms with a compound capable of liberating isoprene by an enzymatic reaction; and (e) detecting the activity of the reporter protein whose expression is induced by sensing isoprene liberated by the enzymatic reaction.

[76] In still another aspect, the present invention also provides a method of quantifying one or more isoprene biosynthesis enzymes activities using an artificial genetic circuit, the method comprising the steps of: (a) providing an artificial genetic circuit for detecting isoprene, the artificial genetic circuit comprising (i) a gene encoding an isoprene-sensing transcriptional regulator which recognizes isoprene, (ii) at least one reporter gene selected from the group consisting of fluorescence protein-encoding genes and antibiotic resistance genes, and (iii) a gene expression regulatory region consisting of a promoter regulating the expression of the isoprene-sensing transcriptional regulator, a region to which the isoprene-sensing transcriptional regulator binds to induce the expression of a downstream reporter gene, and a promoter regulating the expression of the reporter gene; (b) providing a clone or gene library containing one or more of a gene encoding an isoprene biosynthesis enzyme; (c) introducing the clone or gene library and the artificial gene circuit for detecting isoprene into host microorganisms to prepare recombinant microorganisms; (d) treating the recombinant microorganisms with a compound capable of liberating isoprene by an enzymatic reaction; and (e) quantifying the activity of the reporter protein whose expression is induced by sensing isoprene liberated by the enzymatic reaction.

[77] In still another aspect, the present invention also provides a method of quantifying one or more isoprene biosynthesis enzymes using an artificial genetic circuit, the method comprising the steps of: (a) providing microorganisms containing in their chromosomal DNA or cytoplasm an artificial genetic circuit for detecting isoprene, the artificial genetic circuit comprising (i) a gene encoding a isoprene-sensing transcriptional regulator which recognizes isoprene, (ii) at least one reporter gene selected from the group consisting of fluorescence protein-encoding genes and antibiotic resistance genes, and (iii) a gene expression regulatory region consisting of a promoter regulating the expression of the isoprene-sensing transcriptional regulator, a region to which the isoprene-sensing transcriptional regulator binds to induce the expression of a downstream reporter gene, and a promoter regulating the expression of the reporter gene; (b) providing a clone or gene library containing one or more of a gene encoding an isoprene biosynthesis enzyme; (c) introducing the clone or gene library into the microorganisms containing the artificial gene circuit for detecting isoprene to prepare recombinant microorganisms; (d) treating the recombinant microorganisms with a

compound capable of liberating isoprene by an enzymatic reaction; and (e) quantifying the activity of the reporter protein whose expression is induced by sensing isoprene liberated by the enzymatic reaction.

- [78] In the present invention, the reporter gene and the promoter regulating the expression of the reporter gene may be operably linked to each other.
- [79] In the present invention, the region to which the isoprene-sensing transcriptional regulator binds to induce the expression of the downstream reporter gene is a region to which the isoprene-sensing transcriptional regulator binds to activate the promoter of the reporter gene such that the downstream reporter gene can be expressed. The region to which the isoprene-sensing transcriptional regulator binds to activate the promoter of the reporter gene may be OpR (operator for Reporter) region.
- [80] In the present invention, the gene encoding isoprene-sensing transcriptional regulator, which recognizes the isoprene, and the promoter regulating the expression of the isoprene-sensing transcriptional regulator is operably linked to each other.
- [81] In the present invention, the enzymes to be investigated are isoprene biosynthesis enzymes including IspS and MEP/MVA pathway enzymes. For example, Isoprene synthase (IspS), DXP synthase (DXS), DXP reductoisomerase (DXR), CDP-ME synthase (MCT), CDP-ME kinase (CMK), ME-cPP synthase (MDS), HMBPP synthase (HDS), HMBPP reductase (HDR), IPP isomerase (IDI), geranyl diphosphate (GPP), geranylgeranyl diphosphate (GGPP), abscisic acid (ABA), atoB/phaA, mvaS, mvaA, mvaK1, mvaK2 and mvaD. In one embodiment of the present invention, the *Ralstonia pickettii*-derived toluene degradation operon (Tbu operon) regulatory protein TbuT was used.
- [82] In the present invention, the fluorescence protein may be selected from the group consisting of GFP (green fluorescent protein), EGFP (enhanced green fluorescent protein), GFP_{UV} (UV-excited green fluorescent protein), RFP (red fluorescent protein), mRFP (modified red fluorescent protein), YFP (yellow fluorescent protein), mcherry, CFP (cyan fluorescent protein), mGFP (modified green fluorescent protein), ERFP (enhanced red fluorescent protein), BFP (blue fluorescent protein), EBFP (enhanced blue fluorescent protein), EYFP (enhanced yellow fluorescent protein), ECFP (enhanced cyan fluorescent protein) and sfGFP (superfolder green fluorescent protein), and the antibiotic resistance gene may be selected from the group consisting of an ampicillin resistance gene, a kanamycin resistance gene, a chloramphenicol resistance gene and a tetracycline resistance gene.
- [83] In the present invention, the measurement of the activity of the reporter protein is performed using microcolony fluorescence image analysis, fluorescence spectrum analysis, fluorescence-activated cell sorting (FACS), or antibiotic resistance measurement.

- [84] In the present invention, the host microorganism in which the library is introduced is *E. coli*, *Pseudomonas*, a yeast cell, a plant cell, an animal cell or the like.
- [85] In the present invention, the artificial genetic circuit includes a gene coding for RBS (ribosome binding site), and the reporter gene can be a dual reporter gene consisting of a fluorescence protein-encoding gene and an antibiotic resistance gene.
- [86] In the present invention, the “isoprene-sensing transcriptional regulator which recognizes isoprene” can be selected from the genes of XylR, TbuT, TbmR, StyR and TodR. It will be obvious to those skilled in the art that, even when genetically mutated genes thereof are provided according to the present invention, these genetically mutated genes can show the same results. Additionally, any transcriptional regulator, which recognizes isoprene and thus can activate a promoter of a report gene, can be used.
- [87] In the present invention, as shown in FIG. 1B, the “gene expression regulatory region” is a portion regulating the artificial genetic circuit consists of (i) a promoter regulating the expression of the isoprene-sensing transcriptional regulator that is a transcriptional regulator, (ii) a region to which the isoprene-sensing transcriptional regulator (isoprene-sensing transcriptional regulator) binds to induce the expression of a downstream reporter gene, and (iii) a promoter regulating the expression of the reporter gene. When there is no isoprene molecule, domain A of the isoprene-sensing transcriptional regulator inhibits transcription, but when a isoprene molecule binds to inhibit domain A, domains C and D show a function of activating transcription, and thus bind to the OpR (operator for reporter) region as shown in FIG. 1B, and the activity thereof is regulated by depending on σ^{54} .
- [88] As used herein, the term “promoter” means either a promoter regulating the expression of isoprene-sensing transcriptional regulator, or a promoter regulating the expression of the reporter protein. For example, the promoter is a *Ralstonia* *tbuT* or *tbu* operon promoter or a promoter for expression of general protein. For high-level expression of a foreign protein, a high-expression promoter, such as a *trc*, T7, *lac* or *ara* promoter can be used, and particularly, the constitutive high-expression promoter P_{hce} that does not require an inducer can be used. Preferably, the promoter regulating the expression of the reporter gene can be selected from the group consisting of *tbuA* promoter (P_{tbuA}), *tbmA* promoter (P_{tbmA}), Pu promoter and T7 promoter. Additionally, the promoter regulating the expression of the isoprene-sensing transcriptional regulator which recognizes isoprene can be selected from the group consisting of P_{hce} (hyper constitutive expression promoter), P_{ace} (Acetate promoter), P_{Trc} (*trc* promoter), P_{T7} (T7 promoter), P_{lac} (*lac* promoter), P_{ara} (arabinose promoter), BBA_J23105 promoter and BBA_J23114 promoter. It will be obvious to those skilled in the art that, even when genetically modified promoters thereof are provided according to the present invention,

these genetically modified promoters can show the same results.

- [89] Specifically as shown in FIG. 1B, the promoter regulating the expression of the reporter protein is a σ^{54} -dependent promoter (P_R). In addition, a person skilled in the art will appreciate that the promoter is derived from *R. pickettii*, yeast or the like depending on the host of pIspGESS.
- [90] In the present invention, the artificial genetic circuit preferably comprise, in addition to the above promoter, a ribosome binding site (RBS) facilitating the expression of the reporter gene and/or a transcriptional terminator. Namely, the artificial genetic circuit comprise, in addition to the promoter, RBS and/or a transcriptional terminator, which regulates the expression of the regulatory protein.
- [91] Generally, the expression of a protein starts with the initiation codon AUG (methionine) or GUG (Valine) in mRNA, and the discrimination between the protein initiation codons AUG and GUG and the AUG or GUG residue present in the ribosome protein is determined by RBS (or Shine-Dalgarno (SD) sequence) rich in purine bases of DNA, in which RBS is known to be different between species (Stryer, L., (1995) Biochemistry, (4th ed.) W. H. Freeman, Chapter 34, Protein Synthesis). The artificial genetic circuit constructed in the present invention comprises a transcriptional regulator from *Pseudomonas*, which is significantly different from *E. coli*, the host of the genetic circuit. Further, for σ^{54} -dependent gene expression, a σ^{54} -binding site or a σ^{54} -dependent regulator is significantly different from that derived from *E. coli*. Thus, in order to facilitate the expression of the reporter gene in the *Pseudomonas* RBS or the host *E. coli*, *E. coli* RBS or RBS that can be derived from all microbial strains can be used in the present invention. In one embodiment of the present invention, T7 RBS from bacteriophage T7 can be used.
- [92] In the present invention, the transcriptional terminator is preferably rrnBT1T2 or tL3. In addition, any transcriptional terminator that is conventionally used in the art can be used in the present invention.
- [93] In the present invention, one or more reporter gene can be selected from among fluorescence proteins and antibiotic resistance genes. As the fluorescence protein, GFP, GFP_{UV} or RFP is preferably used. In addition, any fluorescence protein can be used so long as it can achieve the object of the present invention. Besides, examples of an antibiotic resistance gene that can be used in the present invention include conventional antibiotic resistance genes, including kanamycin, chloramphenicol, and tetracycline.
- [94] In one embodiment of the present invention, the reporter gene is a dual reporter consisting of both a fluorescence protein and an antibiotic resistance gene, or a multiple reporter consisting of two or more genes. According to the present invention, the metagenomic library and the isoprene-sensing artificial genetic circuit are stepwise transformed into a suitable microbial host. The transformation is carried out using any

known method. In order to increase the efficiency of the transformation, electro-poration is preferably used.

- [95] In the present invention, a gene encoding the enzyme to be screened is provided in the form of a clone or genetic library. For example, it can be provided in the form of a single gene, a genomic library, a metagenome or a metagenomic library, which can be applied in the molecular biological field. In addition, the single gene is provided in a form in which it is contained in a vector or a microorganism.
- [96] In still another aspect, the present invention relates to an artificial genetic circuit for detecting isoprene, and a recombinant microorganism containing the artificial genetic circuit, where in the artificial genetic circuit comprising (i) a gene encoding an isoprene-sensing transcriptional regulator which recognizes isoprene, (ii) at least one reporter gene selected from the group consisting of fluorescence protein-encoding genes and antibiotic resistance genes, and (iii) a gene expression regulatory region consisting of a promoter regulating the expression of isoprene-sensing transcriptional regulator, a region to which the isoprene-sensing transcriptional regulator binds to induce the expression of a downstream reporter gene, and a promoter regulating the expression of the reporter.
- [97] In the present invention, the artificial genetic circuit is provided in the form of a vector or a microorganism. In the present invention, the microorganism is preferably *E. coli*, yeast, a plant cell or an animal cell.
- [98] According to the present invention, the microorganism transformed with the genetic circuit is cultivated to synthesize IspS and to accumulate more DMAPP or IPP in cells.
- [99] The transcriptional regulatory protein is σ^{54} -dependent and operates well in an environment in which nutrient components are limited (Sze *et al.*, (1996) *J. Bacteriol.* 178: 3727-3735). For these reasons, M9 media is more suitable for high-throughput screening than LB media.
- [100] When any enzyme gene is introduced into a recombinant microorganism containing an artificial genetic circuit which senses an isoprene-, toluene- or benzene-based compound and the gene is treated with the isoprene, the concentration of the compound changes depending on the function or activity of the intracellular enzyme gene. Thus, when the quantitative increase of a reporter (such as a fluorescence reporter or an antibiotic resistance reporter), caused by the expression-inducing function of the isoprene, is investigated using various measurement techniques, including fluorescence spectrometry and antibiotic resistance measurement, intracellular and extracellular enzymatic activities can be sensed with high sensitivity. Thus, the present invention provides a novel measurement method of sensing intracellular and extracellular enzymatic activities with high sensitivity.
- [101] Moreover, a fluorescence protein or an antibiotic resistance protein, which is used as

a reporter in the present invention, can be detected by a highly sensitive measurement method and remains in a specific cell without passing through the cell membrane, and individually exhibits the characteristics of a foreign gene which is expressed in the cell. Thus, because a single cell functions as an independent reactor and analyzer, several hundred to several ten million samples can be measured using fluorescence-activated cell sorting (FACS), microcolony fluorescence image analysis, fluorescence spectrum analysis, high-throughput screening with antibiotic selective medium, in order to measure the activity of a reporter whose expression was induced by sensing isoprene released by an enzymatic reaction.

- [102] In still another aspect, the present invention relates to a method of quantifying isoprene using said artificial genetic circuit, the method comprising the steps of: (a) introducing said artificial gene circuit into host microorganisms to be measured; and (b) quantifying isoprene by measuring the activity of the reporter protein whose expression is induced by sensing isoprene.
- [103] In one embodiment of the present invention, IspSm2 having isoprene synthesis activity which is two times higher than that of *Populus trichocarpa*-derived IspSm1 from a patent (USP US2010/000376A1) was isolated by the inventive method of investigating the activity of isoprene biosynthesis enzymes using the artificial genetic circuit.
- [104] In still another aspect, the present invention relates to IspSm2, a mutant IspSm1 containing L35R (SEQ ID NO:8.).
- [105]
- [106] Examples
- [107] Hereinafter, the present invention will be described in further detail with reference to examples. It will be obvious to a person having ordinary skill in the art that these examples are illustrative purposes only and are not to be construed to limit the scope of the present invention.
- [108]
- [109] Example 1: Construction of artificial genetic circuit
- [110] The method for investigating enzymatic activity according to the present invention requires constructing an artificial genetic circuit for sensing isoprene released from a substrate. For this, the GESS genetic circuit (Korean Patent Laid-Open Publication No. 2010-0131955) obtained in previous studies was redesigned, thereby constructing the novel genetic circuit IspGESS(FIG. 1).
- [111] A cloning process was performed in the following manner (FIG. 2). First, a recombinering method was introduced in order to replace the regulatory gene (*dmpR*) and a promoter region for reporter expression with the *R. pickettii*-derived regulatory gene (*tbuT*), based on the GESS plasmid constructed in the previous study (Patent,

KR1020050116672, USP 13/376,783). A promoter for expression of TbuT (P_{hce}) was located at -15 bp from *tbuT* start codon by a restriction enzyme site and an additional sequence. The regulatory gene (*tbuT*) was obtained by colony PCR using a *R. pickettii* strain (ATCC, USA) as a template and primers of SEQ ID NOS. 1 and 2, and the P_{tbuA} promoter region was also obtained by colony PCR using *R. pickettii* as a template and primers of SEQ ID NOS: 3 and 4. Finally, the P_{hce} -TbuT- P_{tbuA} fragment obtained by PCR was amplified by PCR, thereby constructing IspGESS.

[112]

[113] SEQ ID NO: 1 - 5'-gccattagatcttcagcttcgctcgactgga-3'

[114] SEQ ID NO: 2 - 5'-gatatcagctagcccttctggccgcgataagcttgggaa-3'

[115] SEQ ID NO: 3 - 5'-attcttaccattgatgaa-3'

[116] SEQ ID NO: 4 - 5'-cggggtccagttggtcgt-3'

[117]

[118] Example 2: Verification of IspGESS and analysis of quantitative signals for isoprene, toluene, benzene and phenol

[119] Analysis of quantitative signals of artificial genetic circuit for isoprene, toluene, benzene and phenol.

[120] In order to examine the isoprene, toluene, benzene and phenol compounds sensing function of pIspGESS-containing recombinant *E. coli*, pIspGESS of Example 1 was introduced into a single colony of *E. coli* DH5 α (NEB, USA) by heat shock, and then the *E. coli* colony was inoculated into an LB liquid medium (1% (w/v) trypton, 0.5% (w/v) yeast extract, and 1% (w/v) sodium chloride) supplemented with 50 μ g/ml of ampicillin and was cultured with shaking at 37°C for 14 hours. The culture was inoculated into M9 minimal medium (containing 50 μ g/ml ampicillin, 4% glucose and 1% thiamine) at a concentration of 1% and then cultured with shaking at 37°C for 6 hours. Various concentrations (0-2000 μ M) of isoprene was added to each of the test tubes containing the culture broth, after then the culture was cultured with shaking at 30°C for 18 hours, thereby inducing the expression of fluorescence in the culture. The intensity of intracellular fluorescence induced by each concentration of toluene was analyzed by a FACS Calibur system (Becton Dickinson, USA). As a detector, a FSC, SSC, FL1-H (excitation = 488 nm, emission = 530/30 nm) detector was set, and data obtained by observing 10,000 samples were analyzed using CellQuest Pro (Becton Dickinson, USA).

[121] As a result, it was seen that the intensity of fluorescence was definitely distinguished between the presence and absence of isoprene and increased as the isoprene concentration increased. Also the fluorescence intensities increased in proportion to the increases in the concentrations of the substrates (FIG. 3(A)). It was seen that fluorescence started to be sensed from 50 μ M for isoprene.

- [122] Additionally, it was seen that the IspGESS responded to toluene, phenol and benzene (FIGS. 3(B), (C) and (D)). It was seen that fluorescence started to be sensed from 0.5 μ M for toluene, 1 μ M for benzene and 50 μ M for phenol.
- [123] In conclusion, the artificial genetic circuit (IspGESS) constructed by the above-described culture senses quantitatively isoprene, toluene, phenol and benzene and is regulated by them.
- [124]
- [125] Example 3: Detection of isoprene biosynthesis enzyme using IspGESS
- [126] Using the IspGESS genetic circuit according to the present invention, activity of IspSm1, *P. trichocarpa*-derived IspS (USP US2010/000376A1) was sensed.
- [127] First, the IspSm1 gene was inserted into the NcoI-AvrII restriction enzyme site of a pCDF-duet vector (Novagen, USA) to prepare pCDF-IspSm1, which was then introduced into an *E. coli* BL21 (DE3) strain (NEB, USA) containing pIspGESS of Example 1. 50 μ g/ml of ampicillin and 10 μ g/ml of streptomycin were added to LB liquid medium containing 0.1 mM of IPTG (isopropyl-thio- β -Dgalactopyranoside), and *E. coli* cells were cultured with shaking in the LB liquid medium at 37°C for 16 hours. Then, the cultured cells were transferred into M9 liquid medium containing 0.1 mM IPTG and were cultured at 28°C and 200 rpm for 20 hours. 4 ml of the culture medium was placed in a 5 ml vial into which the previously cultured bacterial strain was then inoculated at a concentration of 1%. Then, the vial was sealed with a septum and incubated. The intensity of fluorescence in the cultured cells was analyzed using a FACS Calibur system (Becton Dickinson, USA). As a detector, a FSC, SSC, FL1-H (excitation = 488 nm, emission = 530/30 nm) detector was set, and data obtained by observing 10,000 samples were analyzed.
- [128] As a result, it could be observed that the intensity of fluorescence was higher in the samples containing IspSm1 than in the samples containing no IspSm1 (FIG. 4).
- [129] Based on the above results, a system for quantitative investigation of isoprene, which comprises the IspGESS genetic circuit, was constructed.
- [130]
- [131] Example 4: Screening of isoprene synthase using IspGESS
- [132] Using the artificial genetic circuit, a clone having an improved activity was screened from a library of IspSm1 mutants (FIG. 5). First, a large library was constructed using a random mutagenesis kit (Agilent Technologies, USA). PCR was performed using primers of SEQ ID NOS: 5 and 6, and an average of 5 amino acids in the IspSm1 gene was mutated resultantly. Then, the mutated gene was ligated with pCDF vector as described example 3. Ligation mixture was concentrated using Novagen pellet paint (Millipore, USA) and transformed into an *E. coli* DH5a.
- [133]

[134] SEQ ID NO 5: 5'-cggataacaattcccctgtagaa-3'

[135] SEQ ID NO 6: 5'-tcaagacccggttagaggcc-3'

[136]

[137] As a result, 5×10^5 of mutant library was constructed. The library cells were recovered, and plasmid DNA was isolated from the cells using a plasmid mini prep kit (Qiagen, Germany). Finally, the mutant library DNA was transformed into *E. coli* BL21(DE3) together with the pIspGESS clone by electroporation. To keep the strain healthy, the strain was allowed to stand at 37°C for 1 hour. Then, the cells were applied to LB solid medium containing 50 $\mu\text{g}/\text{mL}$ of ampicillin (pIspGESS internal marker) and 10 $\mu\text{g}/\text{mL}$ of streptomycin (IspSm1 internal marker) and were cultured at 37°C for 16 hours. The number of cell colonies on solid medium was 5×10^5 , and the cell colonies were harvested using storage buffer (1 x TY buffer, 15% (v/v) glycerol, 2% (w/v) glucose). In order to obtain IspSm1 mutant having improved activity, 1×10^4 cells were screened. The library cells were plated on M9 glucose solid medium containing the same amounts of ampicillin and streptomycin as described above and were cultured at 37 °C for 48 hours. With the criterion for screening, cells showing fluorescence intensity which is at least 50% higher than that of IspSm1 were sorted using a FAC-Scalibur system (Becton Dickinson, USA). Then, 6 clones having higher fluorescence intensity than IspSm1 were isolated.

[138] The activities of the isolated clones were analyzed by a FACS. As detectors, FSC, SSC, FL1-H (excitation = 488 nm, emission = 530/30 nm) detectors were set, and 10,000 cells were analyzed. As a result, among the 6 candidates, one mutant IspSm1 showed two times higher fluorescence intensity than that of IspSm1 (FIG. 6).

[139] The screened mutant IspSm2 was sequenced. The nucleotide sequence of the screened IspSm2 is shown by SEQ ID NO: 7, and the amino acid sequence thereof is shown by SEQ ID NO: 8. For the comparison with IspSm2, the nucleotide sequence is SEQ ID NO: 9, and the amino acid sequence is SEQ ID NO: 10. IspSm1 that ligated in pCDF vector is SEQ ID NO: 11, and IspSm2 that ligated in pCDF vector is SEQ ID NO: 12

[140] In FIG. 7, the amino acid sequence of IspSm2 having increased activity contains a lysine-to-arginine mutation at position 35 of the IspSm1 amino acid sequence.

[141] As a result, it was seen that the constructed system can be applied to the large library where cells having improved enzyme activity could be found.

[142]

[143] Example 5: Application of IspGESS with another vector system for IspS expression

[144] For another experiment, using the IspGESS genetic circuit IspS expression vector was changed to pPROLar A122 (clonetechn, USA).

[145] First, IspSm1 and IspSm2 genes were amplified using PCR with primers (SEQ ID

NOS: 13, 14), and inserted into the NdeI-XbaI restriction enzyme site of a pPROLar vector for pPROLar-ispSm1 and pPROLar-ispSm2 (SEQ ID NOS: 15, 16). Then these two vectors were transformed into an *E. coli* DH5 α strain containing IspGESS of Example 1. 50 $\mu\text{g}/\text{ml}$ of ampicillin and 10 $\mu\text{g}/\text{ml}$ of kanamycin were added to LB liquid medium, and *E. coli* cells were cultured with shaking in the LB liquid medium at 37°C for 16 hours. 4 ml of the culture medium was placed in a 10ml vial and the cultured bacterial strain was then inoculated at a concentration of 1%. Then, the vial was sealed with a septum and the cells were cultured at 28°C and 200 rpm for 24 hours. The intensity of fluorescence in the cultured cells was analyzed using a FACScalibur system (Becton Dickinson, USA). As detectors, FSC, SSC, and FL1-H (excitation = 488 nm, emission = 530/30 nm) detectors were set, and 10,000 samples were analyzed.

[146] As a result, it could be observed that the intensity of fluorescence was higher in the cells containing IspSm1 or IspSm2 than in the cells containing neither IspSm1 nor IspSm2 (FIG. 6).

[147] To verify the fluorescence differences of IspSm1, IspSm2 and no IspS induction control, the flow cytometry data was analyzed by t-test. The t-test compared the mean values of the fluorescence signals between the negative control (IspGESS without IspSm1 (IspSm2) induction) and the case that includes IspSm1 (IspSm2) inducer. The result showed a significant difference of their activities (p-value = 0.0006 (0.0178)) despite of small replicates. Note that there are three replicates of each case (FIG. 8). Additionally, IspGESS with IspSm2 shows higher fluorescent mean intensity than that of IspGESS with IspSm1.

[148]

[149] Example 6: Improved isoprene sensitivity by control the regulator expression

[150] To confirm the sensitivity of the TbuT regulator expression system, we changed the HCE promoter to TRC and ACE promoters, respectively. First, pIspGESS of Example 1 digested by *NheI* and *ClaI* restriction enzymes to subclone TRC promoter (SEQ ID NO 17) or ACE promoter (SEQ ID NO 18). Second, TRC or ACE promoter was amplified to ligate into pIspGESS, and also digested by *NheI* and *ClaI* restriction enzymes. For the sensing function of pIspGESS-containing recombinant *E. coli*, pIspGESS_{ACE} or pIspGESS_{TRC} was introduced into a single colony of *E. coli* DH5 α by heat shock. Then the *E. coli* colony were inoculated into an LB liquid medium (1% (w/v) trypton, 0.5% (w/v) yeast extract, and 1% (w/v) sodium chloride) supplemented with 50 $\mu\text{g}/\text{ml}$ of ampicillin and was cultured with shaking at 37°C for 14 hours. The culture was inoculated into M9 minimal medium (containing 50 $\mu\text{g}/\text{ml}$ ampicillin, 4% glucose and 1% thiamine) at a concentration of 1% and then cultured with shaking at 37°C for 6 hours. 100 μM of isoprene was added to each of the test tubes containing the culture broth, after then the culture was incubated with shaking at 27°C for 18

hours, thereby inducing the expression of fluorescence induced by the isoprene in the culture. The intensity of the intracellular fluorescence was analyzed by a FACS Calibur system (Becton Dickinson, USA). As a detector, a FSC, SSC, FL1-H (excitation = 488 nm, emission = 530/30 nm) detector was set, and data obtained by observing 10,000 samples were analyzed using CellQuest Pro (Becton Dickinson, USA).

[151] As a result, intensity of reporter for pIspGESS_{ACE} was similar to the pIspGESS_{HCE}. However, the reporter intensity of pIspGESS_{TRC} increased 5 times better than the best results achieved with pIspGESS_{HCE} (14.69→82.4) (FIG. 10).

[152]

[153] Example 7: Construction of IspGESSTRC-sfGFP and Evaluation

[154] A recombineering method was applied in order to construct the plasmid, IspGESS_{TRC}_sfGFP based on the earlier construct IspGESS_{TRC}. The pIspGESS_{TRC}_sfGFP was constructed by swapping out the EGFP gene of pIspGESS_{TRC} with the sfGFP (Gene accession No. HQ873313.1, Nature biotechnology, 24:79-88, 2006). The sfGFP gene was amplified by PCR using the plasmid pHCEIIB-sfGFP as template and the primers of SEQ ID NOS. 19 and 20. The IspGESS back bone was also obtained by PCR using pIspGESS as template and the primers of SEQ ID NOS. 21 and 22. The amplified PCR products were ligated by Gibson assembly and the recombinant plasmid was introduced into a single colony of *E. coli* DH5α cells by electroporation.

[155]

[156] SEQ ID NO: 19- cctgattaactttataaggaggaaaaacatatgagcaaagggtgaagaactgtttaccggc

[157] SEQ ID NO: 20- aaatcttctctcatccgccccaaacagaagcttattagtgatggatgatgagaacc

[158] SEQ ID NO: 21- ggttctcatcaccatcaccatcactaataagcttctgtttggcggatgagagaagattt

[159] SEQ ID NO: 22- gccggtaaacagttcttcacctttgctcatatgttttctctctataaagttaatcagg

[160]

[161] In order to compare the efficacy of IspGESS_{TRC}_sfGFP over IspGESS_{TRC}_EGFP, a single colony of *E. coli* DH5α cells harboring either pIspGESS_{TRC}_EGFP or pIspGESS_{TRC}_sfGFP were inoculated into M9 minimal medium (containing 0.4% glucose, 0.5 μg/ml thiamine, and 50 μg/ml ampicillin) and then cultivated aerobically at 37°C with a shaking of 200 rpm for 16 hours. This culture was used to inoculate the main culture (2 ml/15 ml serum bottle) to an OD₆₀₀ of 0.05, and induced with varying concentration of isoprene ranging from 0 to 2000 μM. The culture bottles were sealed airtight using butyl septum, incubated at 28°C for 24 hours with a shaking of 200 rpm, thereby inducing the expression of fluorescence protein in the cells. The intensity of the intracellular fluorescence induced by each concentration of isoprene was analyzed by FACS Calibur system (Becton Dickinson, USA). As a detector, a FSC, SSC, FL1-H (excitation = 488 nm, emission = 530/30 nm) detector was set, and data obtained

by observing 10,000 samples were analyzed using CellQuest Pro (Becton Dickinson, USA).

- [162] The fluorescence intensity of the cells harbouring pIspGESS_{TRC}-sfGFP was higher compared to those harbouring pIspGESS_{TRC}-EGFP, with each concentration of isoprene (Figure 11). The fluorescence intensity with sfGFP showed a fold increase of 2.2 compared to that with EGFP. A fold increase of 2.2 was calculated with the fluorescence intensity of sfGFP compared to that of EGFP.

[163]

- [164] Example 8: Applications for improving isoprene sensitivity of IspGESS system

- [165] Construction of BL21(DE3)*PtbuA1*:T7RNAP

- [166] A applied double strand break method (WO2013154312 A1) was applied in order to construct BL21(DE3)*PtbuA1*:T7RNAP for scarless switch of variation. The Sequences of chloramphenicol acetyltransferase gene with I-SceI enzyme recognition sites on either side are amplified by PCR using the plasmid pKD3-IscE (WO2013154312 A1) as template and the primers of SEQ ID NOS. 23 and 24, and the sequences are 1st inserted into promoter region of T7 RNA polymerase of BL21(DE3).

- [167] SEQ ID NO:

23-5'-ccaccctggcgcccaatacgcgaaccgcctctccccgcgcgggtccatgaatactctcc-3': lacUV5KO-F

- [168] SEQ ID NO: 24- 5'-gcgatgttaatcggttcatttagtgcctctccagttagggaatacggtagccatttg-3')
Using: lacUV5KO-R

- [169] For 2nd recombination, The *rrnB* terminator for preventing Read-through transcription into T7 RNA polymerase gene of near region and the sequences of *PtbuA1* promoter region for promoter of T7 RNA polymerase are ligated and synthesized the sequences(Bioneer, Inc, Korea).

- [170] The synthesized sequences are amplified by PCR Using the primers of SEQ ID NOS. 25 and 26, and constructed BL21(DE3)*PtbuA1*:T7RNAP by 2nd Recombination. The inserted sequences of constructed cells were determined by sequencing.

[171]

- [172] SEQ ID NO: 25- 5'-cgacttatgccccgagaagat-3': TP-F

- [173] SEQ ID NO: 26- 5'-gagctaaacgctcaccgtaa-3': TP-R

[174]

- [175] Construction of T7IspGESS_{TRC} Plasmid

- [176] *PtbuA1*(GFP promoter) of IspGESS_{TRC} was deleted by enzymatic digestion with *Bam*HI and *Kpn*I. A dsDNA containing T7 promoter was constructed by annealing with primers SEQ ID NOS. 27 and 28, and it is cloned in site of *Bam*HI and *Kpn*I of the pIspGESS_{TRC}.

- [177] Finally, the cloned plasmid sequences were determined by sequencing.

[178]

[179] SEQ ID NO: 27- 5'- ctaatacgactcactatagggg -3': T7PA-F

[180] SEQ ID NO: 28- 5'- gatccccctatagtgcgtattaggtag -3': T7PA-R

[181]

[182] Construction of T7IspGESS_{HCE} Plasmid

[183] *PtbuAI*(GFP promoter) of pIspGESS_{HCE} was deleted by enzymatic digestion with *Bam*HI and *Kpn*II. A dsDNA containing T7 promoter was constructed by annealing with primers SEQ ID NOS. 27 and 28, and it is cloned in site of *Bam*HI and *Kpn*II of the pIspGESS_{HCE}.

[184] Finally, the cloned plasmid sequences were determined by sequencing.

[185]

[186] Construction of T7IspGESS_{TRC}_sfGFP Plasmid

[187] The sfGFP gene was obtained by method of Example 7 using primers SEQ ID NOS. 29 and 30, and pIspGESS_{TRC} was amplified by PCR using primers SEQ ID NOS. 31 and 32. The two PCR products are ligated by Gibson assembly, and T7IspGESS_{TRC}_sfGFP was constructed.

[188]

[189] SEQ ID NO: 29- 5'- cgaaaaataagcttctgttttggcggatga -3': GibV-F

[190] SEQ ID NO: 30- 5'- ctttgctcatatgttttctctctataaa -3': GibV-R

[191] SEQ ID NO: 31- 5'- ggaaaaacatatgagcaaaggtgaagaact -3': GibI-F

[192] SEQ ID NO: 32- 5'- gatccccctatagtgcgtattaggtag -3': GibI-R

[193]

[194] Construction of T7IspGESS_{J23105} Plasmid

[195] *Trc* (super folder GFP promoter) of T7IspGESS_{TRC}_sfGFP was deleted by enzymatic digestion with *Nco*II and *Cla*II. A dsDNA containing J23105 promoter(SEQ ID NOS. 33) was constructed by annealing with primers SEQ ID NOS. 34 and 35, and it is cloned in site of *Nco*II and *Cla*II of the T7IspGESSTRC_sfGFP(figure 12).

[196]

[197] SEQ ID NOS. 33- 5'- ttacggctagctcagtcctaggtactatgctagc -3'

[198]

[199] Finally, the cloned plasmid sequences were determined by sequencing.

[200]

[201] SEQ ID NO: 34- 5'- catggtttctctctttaatctctagtagcatagtagctagtagccgtaaaat -3': J23105-F

[202] SEQ ID NO: 35- 5'- cgattttacggctagctcagtcctaggtactatgctagctactagagattaaa-gaggagaaac -3': J23105-R

[203]

[204] Construction of T7IspGESS_{J23114} Plasmid

[205] *Trc*(super folder GFP promoter) of T7IspGESS_{TRC}_sfGFP was deleted by enzymatic

digestion with *NcoI* and *ClaI*. A dsDNA containing J23114 promoter(SEQ ID NOS. 36) was constructed by annealing with primers SEQ ID NOS. 37 and 38, and it is cloned in site of *NcoI* and *ClaI* of the T7IspGESS_{TRC}-sfGFP(figure 12).

[206]

[207] SEQ ID NOS. 36- 5'- tttatggctagctcagtcctaggtacaatgctagc-3'

[208] Finally, the cloned plasmid sequences were determined by sequencing.

[209]

[210] SEQ ID NO: 37- 5'- catggtttctcctctttaatctctagtagctagcattgtacctaggactgagctagccataaaat-3': J23114-F

[211] SEQ ID NO: 38- 5'- cgattttatggctagctcagtcctaggtacaatgctagctactagagattaaa-gaggagaaac -3': J23114-R

[212]

[213] Example 9: Verification of constructed IspGESS plasmids: IspGESS_{TRC}, IspGESS_{HCE}, T7IspGESS_{TRC} and T7IspGESS_{HCE}

[214] In order to compare the efficacy of IspGESS_{TRC}, IspGESS_{HCE}, T7IspGESS_{TRC} and T7IspGESS_{HCE}, a single colony of *E. coli* BL21(DE3)*PtbaA1*:T7RNAP cells respectively harboring of IspGESS_{TRC}, IspGESS_{HCE}, T7IspGESS_{TRC} and T7IspGESS_{HCE}, were inoculated into M9 minimal medium (containing 0.4% glucose, 0.5 µg/ml thiamine, and 50 µg/ml ampicillin) and then cultivated aerobically at 30°C with a shaking of 200 rpm for 16 hours. This culture was used to inoculate the main culture (2 ml/15 ml serum bottle) to an OD₆₀₀ of 0.05, and induced with concentration of isoprene 0 or 1000 µM. The culture bottles were sealed airtight using butyl septum, incubated at 30°C for 6 hours with a shaking of 200 rpm, thereby inducing the expression of fluorescence protein in the cells. The intensity of the intracellular fluorescence induced by each concentration of isoprene was analyzed at O.D.600 by fluorometer(Perkin Elmer, USA).

[215] The fluorescence intensity of the cells harbouring T7IspGESS_{TRC} was higher compared to another cells, with each concentration of isoprene (Figure 13 A). The fluorescence intensity with T7IspGESSTRC added with 1 mM isoprene showed a fold increase of 8.9 compared to that with T7IspGESSTRC without addition of isoprene.

[216]

[217] Example 10: Verification of T7IspGESS_{TRC} plasmids with various concentration of isoprene

[218] For verification of T7IspGESS_{TRC} with various rage of isoprene, a single colony of *E. coli* BL21(DE3)*PtbaA1*:T7RNAP cells harboring of T7IspGESS_{TRC} were inoculated into M9 minimal medium (containing 0.4% glucose, 0.5 µg/ml thiamine, and 50 µg/ml ampicillin) and then cultivated aerobically at 30°C with a shaking of 200 rpm for 16 hours. This culture was used to inoculate the main culture (2 ml/15 ml serum bottle) to

an OD₆₀₀ of 0.05, and induced with varying concentration of isoprene ranging from 0, 25, 50, 100 and 1000 μ M. The culture bottles were sealed airtight using butyl septum, incubated at 30°C for 6 hours with a shaking of 200 rpm, thereby inducing the expression of fluorescence protein in the cells. The intensity of the intracellular fluorescence induced by each concentration of isoprene was analyzed at O.D.600 by fluorometer(Perkin Elmer, USA).

[219] The induction fold with varying concentration of isoprene of T7IspGESSTRC was shows Figure 13B.

[220]

[221] Example 11: Verification of constructed IspGESS plasmids: IspGESS_{TRC}, T7IspGESS_{TRC} and T7IspGESS_{TRC_sfGFP}

[222] In order to compare the efficacy of IspGESS_{TRC}, T7IspGESS_{TRC} and T7IspGESS_{TRC_sfGFP}, a single colony of *E. coli* BL21(DE3)*PtbaA1*:T7RNAP cells respectively harboring of IspGESSTRC, T7IspGESS_{TRC} and T7IspGESS_{TRC_sfGFP}, were inoculated into M9 minimal medium (containing 0.4% glucose, 0.5 μ g/ml thiamine, and 50 μ g/ml ampicillin) and then cultivated aerobically at 30°C with a shaking of 200 rpm for 16 hours. This culture was used to inoculate the main culture (2 ml/15 ml serum bottle) to an OD₆₀₀ of 0.05, and induced with concentration of isoprene 0 or 1000 μ M. The culture bottles were sealed airtight using butyl septum, incubated at 30°C for 6 hours with a shaking of 200 rpm, thereby inducing the expression of fluorescence protein in the cells. The intensity of the intracellular fluorescence induced by each concentration of isoprene was analyzed at O.D.600 by fluorometer(Perkin Elmer, USA).

[223] The fluorescence intensity of the cells harbouring T7IspGESS_{TRC_sfGFP} was higher compared to another cells, with each concentration of isoprene (Figure 14). The fluorescence intensity with T7IspGESS_{TRC_sfGFP} showed a induction fold of 26.4 when added 1 mM isoprene.

[224]

[225] Example 12: Verification of constructed IspGESS plasmids: T7IspGESS_{TRC_sfGFP}, T7IspGESS_{J23105_sfGFP} and T7IspGESS_{J23114_sfGFP}

[226] In order to compare the efficacy of T7IspGESS_{TRC_sfGFP}, T7IspGESS_{J23105_sfGFP} and T7IspGESS_{J23114_sfGFP}, a single colony of *E. coli* BL21(DE3)*PtbaA1*:T7RNAP cells respectively harboring of T7IspGESS_{TRC_sfGFP}, T7IspGESS_{J23105_sfGFP} and T7IspGESS_{J23114_sfGFP}, were inoculated into M9 minimal medium (containing 0.4% glucose, 0.5 μ g/ml thiamine, and 50 μ g/ml ampicillin) and then cultivated aerobically at 30°C with a shaking of 200 rpm for 16 hours. This culture was used to inoculate the main culture (2 ml/15 ml serum bottle) to an OD₆₀₀ of 0.05, and induced with concentration of isoprene 0 or 1000 μ M. The culture bottles were sealed airtight using butyl septum, incubated at 30°C for 6 hours with a shaking of 200 rpm, thereby

inducing the expression of fluorescence protein in the cells. The intensity of the intra-cellular fluorescence induced by each concentration of isoprene was analyzed at O.D.600 by fluorometer(Perkin Elmer, USA).

[227] The fluorescence intensity of the cells harbouring T7IspGESS_{J23105}_sfGFP was higher compared to another cells, with each concentration of isoprene (Figure 15). The fluorescence intensity with T7IspGESS_{J23105}_sfGFP showed a induction fold of 20.7 when added with 1.0 mM isoprene.

[228]

Industrial Applicability

[229] As described above, when the inventive method for screening and quantifying target enzymatic activity is used, the high sensitivity of the genetic circuit to isoprene enables to sense a small amount of isoprene synthase in a single cell rapidly so that it could be possible to find a novel isoprene biosynthesis enzymes from the large library for the efficient and mass production of isoprene. Also the invented method can measure the enzymatic activity quantitatively, which enables to engineering the proteins in MEP/MVA pathway for the improvement of the isoprene production in the microorganism.

[230] More generally, the invention can be advantageously used in the protein engineering technology and large-scale screening for industrially valuable biocatalysts.

[231] Although the present invention has been described in detail with reference to the specific features, it will be apparent to those skilled in the art that this description is only for a preferred embodiment and does not limit the scope of the present invention. Thus, the substantial scope of the present invention will be defined by the appended claims and equivalents thereof.

[232]

Sequence Listing Free Text

[233] Electronic document attached.

Claims

[Claim 1]

A method of screening one or more isoprene biosynthesis enzymes' activities using an artificial genetic circuit, the method comprising the steps of:

- (a) providing an artificial genetic circuit for detecting isoprene, the artificial genetic circuit comprising (i) a gene encoding an isoprene-sensing transcriptional regulator which recognizes isoprene, (ii) at least one reporter gene selected from the group consisting of fluorescence protein-encoding genes and antibiotic resistance genes, and (iii) a gene expression regulatory region consisting of a promoter regulating the expression of the isoprene-sensing transcriptional regulator, a region to which the isoprene-sensing transcriptional regulator binds to induce the expression of a downstream reporter gene, and a promoter regulating the expression of the reporter gene;
- (b) providing a clone or gene library containing one or more of a gene encoding an isoprene biosynthesis enzyme;
- (c) introducing the clone or gene library and the artificial gene circuit for detecting isoprene into host microorganisms to prepare recombinant microorganisms;
- (d) treating the recombinant microorganisms with a compound capable of liberating isoprene by an enzymatic reaction; and
- (e) detecting the activity of the reporter protein whose expression is induced by sensing isoprene liberated by the enzymatic reaction.

[Claim 2]

A method of screening one or more isoprene biosynthesis enzymes' activities using an artificial genetic circuit, the method comprising the steps of:

- (a) providing microorganisms containing in their chromosomal DNA or cytoplasm an artificial genetic circuit for detecting isoprene, the artificial genetic circuit comprising (i) a gene encoding an isoprene-sensing transcriptional regulator which recognizes isoprene, (ii) at least one reporter gene selected from the group consisting of fluorescence protein-encoding genes and antibiotic resistance genes, and (iii) a gene expression regulatory region consisting of a promoter regulating the expression of the isoprene-sensing transcriptional regulator, a region to which the isoprene-sensing transcriptional regulator binds to induce the expression of a downstream reporter gene, and a promoter regulating the expression of the reporter gene;

- (b) providing a clone or gene library containing one or more of a gene encoding an isoprene biosynthesis enzyme;
- (c) introducing the clone or gene library into the microorganisms containing the artificial gene circuit for detecting isoprene to prepare recombinant microorganisms;
- (d) treating the recombinant microorganisms with a compound capable of liberating isoprene by an enzymatic reaction; and
- (e) detecting the activity of the reporter protein whose expression is induced by sensing isoprene liberated by the enzymatic reaction.

[Claim 3]

A method of quantifying one or more isoprene biosynthesis enzymes activities using an artificial genetic circuit, the method comprising the steps of:

- (a) providing an artificial genetic circuit for detecting isoprene, the artificial genetic circuit comprising (i) a gene encoding an isoprene-sensing transcriptional regulator which recognizes isoprene, (ii) at least one reporter gene selected from the group consisting of fluorescence protein-encoding genes and antibiotic resistance genes, and (iii) a gene expression regulatory region consisting of a promoter regulating the expression of the isoprene-sensing transcriptional regulator, a region to which the isoprene-sensing transcriptional regulator binds to induce the expression of a downstream reporter gene, and a promoter regulating the expression of the reporter gene;
- (b) providing a clone or gene library containing one or more of a gene encoding an isoprene biosynthesis enzyme;
- (c) introducing the clone or gene library and the artificial gene circuit for detecting isoprene into host microorganisms to prepare recombinant microorganisms;
- (d) treating the recombinant microorganisms with a compound capable of liberating isoprene by an enzymatic reaction; and
- (e) quantifying the activity of the reporter protein whose expression is induced by sensing isoprene liberated by the enzymatic reaction.

[Claim 4]

A method of quantifying one or more isoprene biosynthesis enzymes using an artificial genetic circuit, the method comprising the steps of:

- (a) providing microorganisms containing in their chromosomal DNA or cytoplasm an artificial genetic circuit for detecting isoprene, the artificial genetic circuit comprising (i) a gene encoding an isoprene-sensing transcriptional regulator which recognizes isoprene, (ii) at least one reporter gene selected from the group consisting of fluorescence

protein-encoding genes and antibiotic resistance genes, and (iii) a gene expression regulatory region consisting of a promoter regulating the expression of the isoprene-sensing transcriptional regulator, a region to which the isoprene-sensing transcriptional regulator binds to induce the expression of a downstream reporter gene, and a promoter regulating the expression of the reporter gene;

(b) providing a clone or gene library containing one or more of a gene encoding an isoprene biosynthesis enzyme;

(c) introducing the clone or gene library into the microorganisms containing the artificial gene circuit for detecting isoprene to prepare recombinant microorganisms;

(d) treating the recombinant microorganisms with a compound capable of liberating isoprene by an enzymatic reaction; and

(e) quantifying the activity of the reporter protein whose expression is induced by sensing isoprene liberated by the enzymatic reaction.

[Claim 5]

The method of any one claim among claims 1 to 4, wherein the compound capable of liberating isoprene is selected from the group consisting of dimethylallyl pyrophosphate (DMAPP), methylcyclohexane, heptane, 1-deoxy-D-xylulose 5-phosphate (DXP), 2-C-methyl-D-erythritol 4-phosphate (MEP), 4-(cytidine 5'-diphospho)-2-C-methyl-D-erythritol, 2-phospho-4-(cytidine 5'-di-phospho)-2-C-methyl-D-erythritol (CDP-MEP), 2-C-methyl-D-erythritol 2,4-cyclodiphosphate (ME-cPP), 4-hydroxy-3-methylbut-2-enyl diphosphate (HMBPP), isopentenyl diphosphate (IPP), Acetoacetyl-CoA, HMG-CoA, Mevalonate, phosphomevalonate and diphosphomevalonate.

[Claim 6]

The method of any one claim among claims 1 to 4, wherein the region to which the isoprene-sensing translator regulator binds to induce the expression of reporter gene is a region to which the isoprene-sensing translator regulator binds to activate the promoter of the reporter gene such that the downstream reporter gene can be expressed.

[Claim 7]

The method of any one claim among claims 1 to 4, wherein the reporter gene and the promoter regulating the expression of the reporter gene are operably linked to each other.

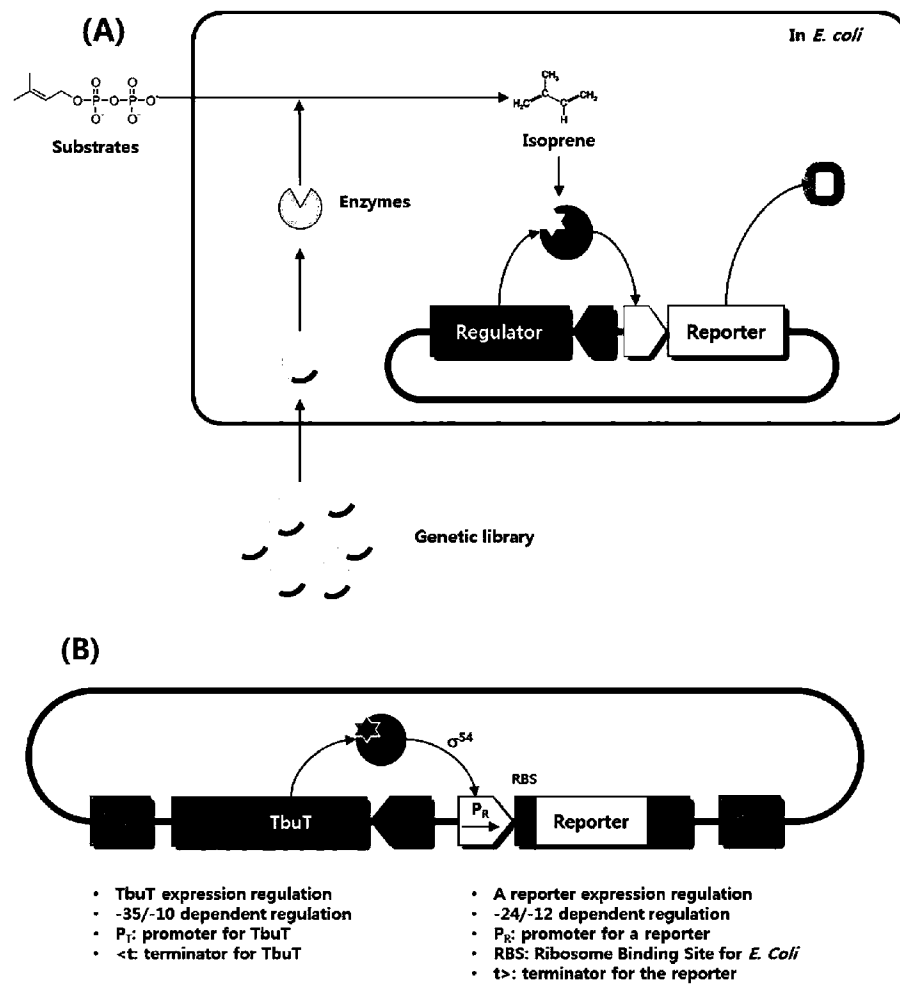
[Claim 8]

The method of claim 7, wherein the promoter regulating the expression of the reporter gene is selected from the group consisting of *tbuA* promoter (Pt_{buA}), *tbmA* promoter (Pt_{bmA}), Pu promoter (Pu), Pr promoter (Pr) and T7 promoter.

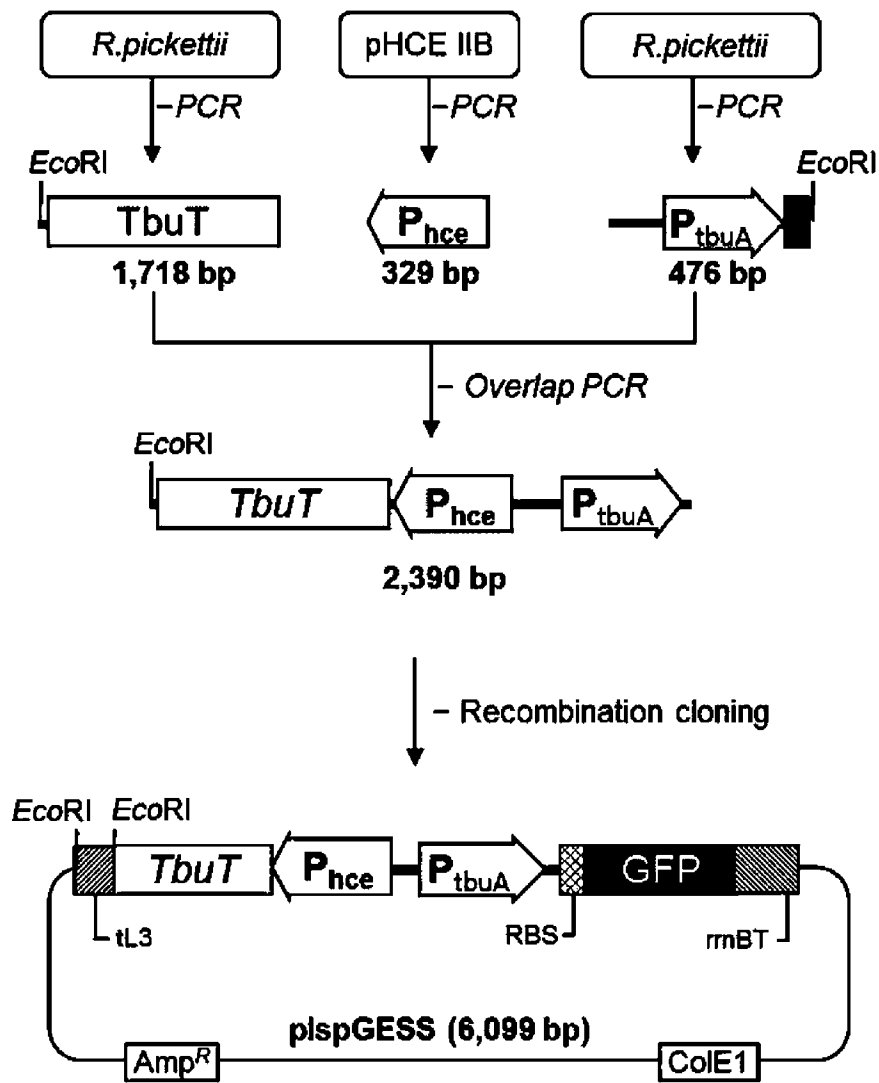
- [Claim 9] The method of any one claim among claims 1 to 4, wherein the isoprene biosynthesis enzyme is selected from the group consisting of Isoprene synthase (IspS), DXP synthase (DXS), DXP reductoisomerase (DXR), CDP-ME synthase (MCT), CDP-ME kinase (CMK), ME-cPP synthase (MDS), HMBPP synthase (HDS), HMBPP reductase (HDR), IPP isomerase (IDI), geranyl diphosphate (GPP), geranylgeranyl diphosphate (GGPP), abscisic acid (ABA), atoB/phaA, mvaS, mvaA, mvaK1, mvaK2 and mvaD.
- [Claim 10] The method of any one claim among claims 1 to 4, wherein the isoprene-sensing transcriptional regulator which recognizes isoprene is selected from the group consisting of TbuT, XylR, TbmR, StyR and TodR.
- [Claim 11] The method of any one claim among claims 1 to 4, wherein the gene encoding a isoprene-sensing transcriptional regulator which recognizes isoprene and the promoter regulating the expression of the regulatory protein are operably linked to each other.
- [Claim 12] The method of any one claim among claims 1 to 4, wherein the promoter regulating the expression of the isoprene-sensing transcriptional regulator which recognizes isoprene is selected from the group consisting of P_{hce}(hyper constitutive expression promoter), P_{ace}(Acetate promoter), P_{Trc}(trc promoter), P_{T7}(T7 promoter), P_{lac}(lac promoter), P_{ara}(arabinose promoter), BBa_J23105 promoter and BBa_J23114 promoter.
- [Claim 13] The method of any one claim among claims 1 to 4, wherein the fluorescence protein is selected from the group consisting of GFP (green fluorescent protein), EGFP (enhanced green fluorescent protein), GFP_{UV}(UV-excited green fluorescent protein), RFP(red fluorescent protein), mRFP(modified red fluorescent protein), YFP(yellow fluorescent protein), mcherry, CFP(cyan fluorescent protein), mGFP(modified green fluorescent protein), ERFP(enhanced red fluorescent protein), BFP(blue fluorescent protein), EBFP(enhanced blue fluorescent protein), EYFP(enhanced yellow fluorescent protein), ECFP(enhanced cyan fluorescent protein) and sfGFP(superfolder green fluorescent protein).
- [Claim 14] The method of any one claim among claims 1 to 4, wherein the reporter gene is multiple reporter genes consisting of two or more reporter genes selected from among fluorescence protein-encoding genes and antibiotic resistance genes.

- [Claim 15] The method of any one claim among claims 1 to 4, wherein the antibiotic resistance gene is selected from the group consisting of ampicillin, kanamycin, streptomycin, chloramphenicol and tetracycline.
- [Claim 16] The method of any one claim among claims 1 to 4, wherein the measuring the activity of the reporter protein is performed by using a method selected from the group consisting of microcolony image analysis, fluorescence spectrum analysis, fluorescence-activated cell sorting (FACS), and antibiotic resistance measuring method.
- [Claim 17] The method of any one claim among claims 1 to 4, wherein the microorganism is selected from the group consisting of *E. coli*, *Ralstonia*, yeast, plant cells and animal cells.
- [Claim 18] The method of any one claim among claims 1 to 4, wherein the artificial genetic circuit contains a gene encoding RBS(ribosome binding site).
- [Claim 19] An artificial genetic circuit for detecting isoprene, the artificial genetic circuit comprising (i) a gene encoding an isoprene-sensing transcriptional regulator which recognizes isoprene, (ii) at least one reporter gene selected from the group consisting of fluorescence protein-encoding genes and antibiotic resistance genes, and (iii) a gene expression regulatory region consisting of a promoter regulating the expression of the isoprene-sensing transcriptional regulator, a region to which the isoprene-sensing transcriptional regulator binds to induce the expression of a downstream reporter gene, and a promoter regulating the expression of the reporter gene.
- [Claim 20] A recombinant microorganism containing the artificial genetic circuit of the claim 19.
- [Claim 21] A method of quantifying isoprene using the artificial genetic circuit of claim 18, the method comprising the steps of:
(a) introducing the artificial gene circuit of claim 19 into host microorganisms to be measured; and
(b) quantifying isoprene by measuring the activity of the reporter protein whose expression is induced by sensing isoprene.
- [Claim 22] A gene encoding IspSm2 of SEQ ID NO: 8.

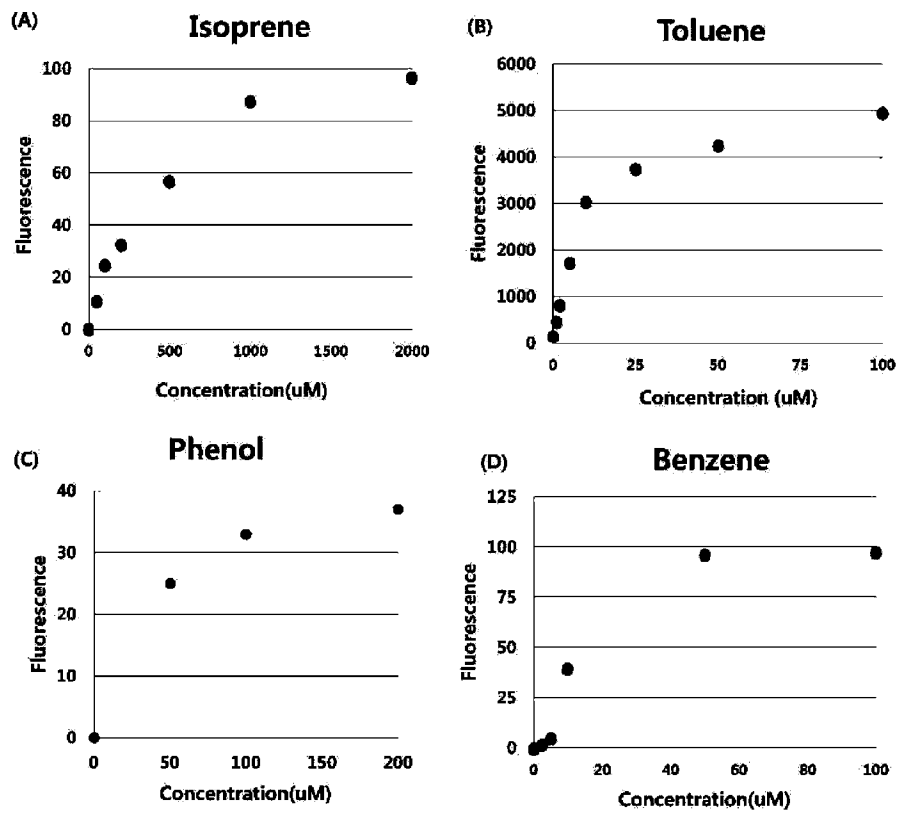
[Fig. 1]



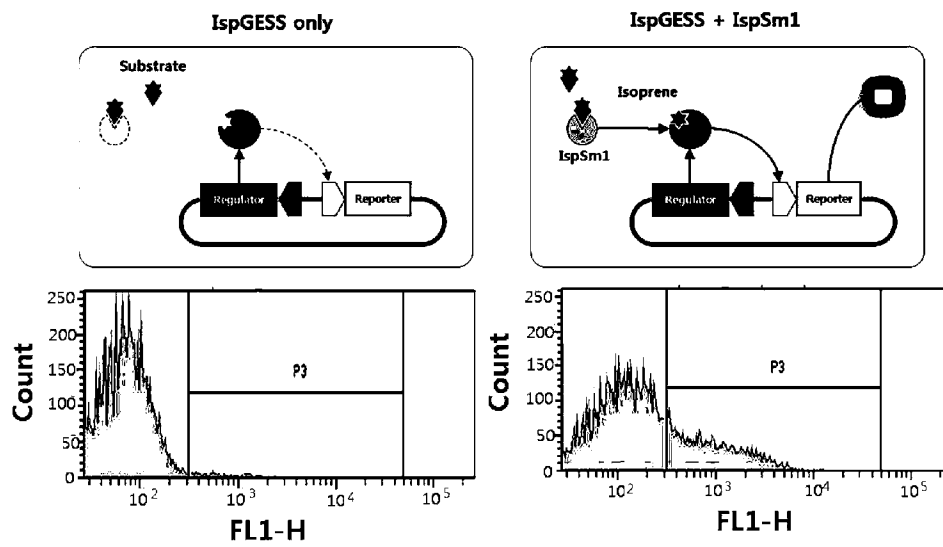
[Fig. 2]



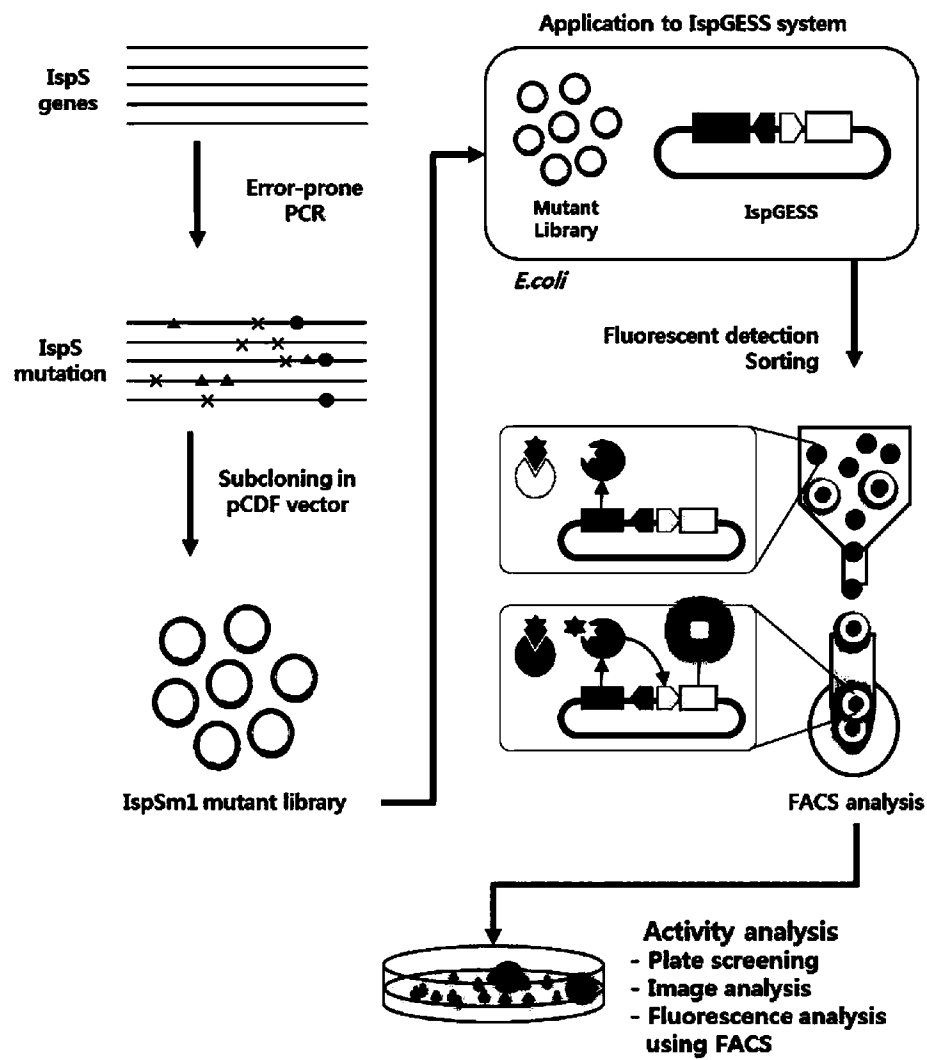
[Fig. 3]



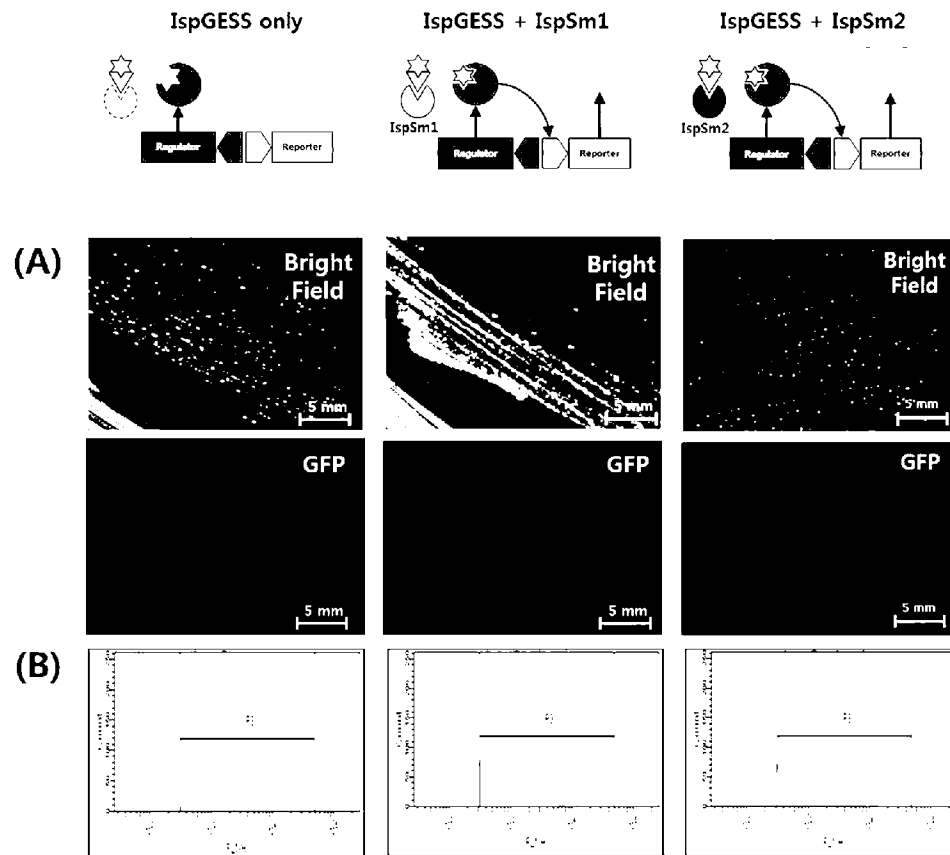
[Fig. 4]



[Fig. 5]



[Fig. 6]



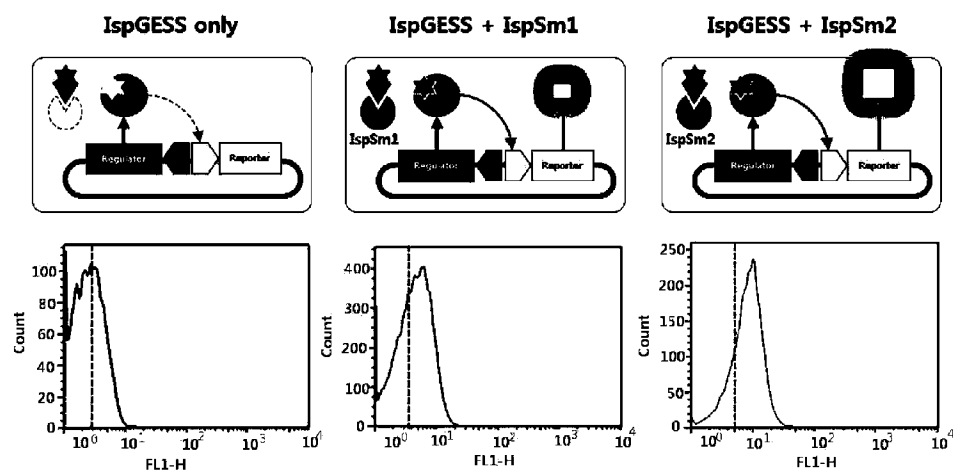
[Fig. 7]

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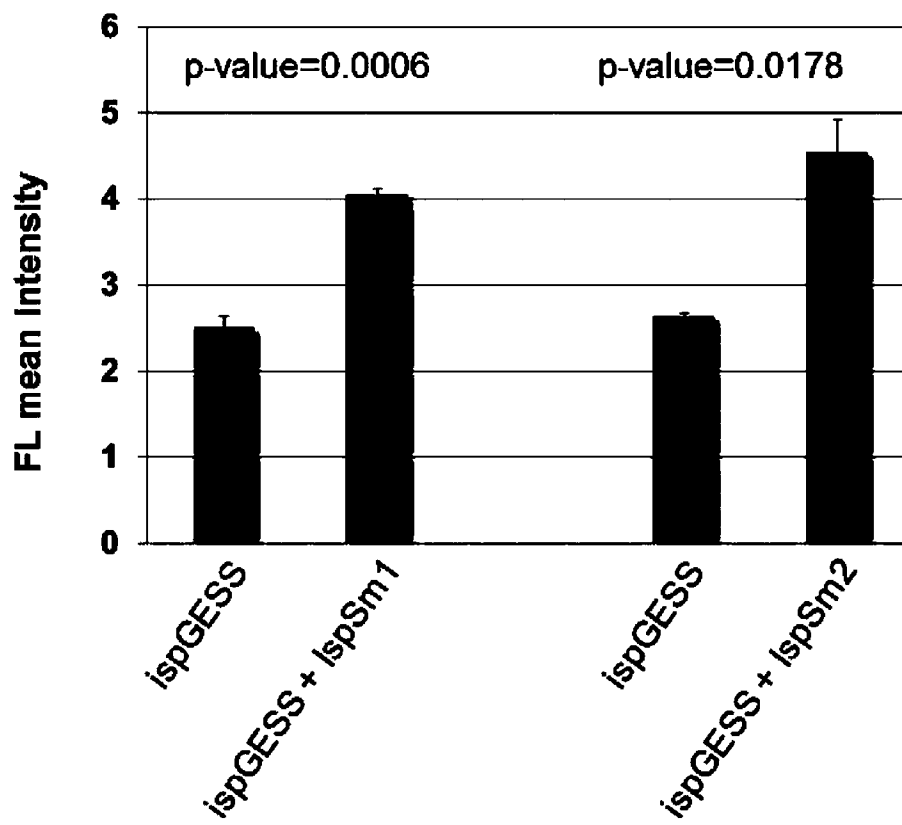
IspSm1 : MACSVSTENVSTTETETETRRSANYEPNSWDYDYLSSDTDESIEVYKDKAKKLEAEVRRBINNEKAEFTLLELDNVQRLGLGYRFESDIRRALDRFY : 100
IspSm2 : MACSVSTENVSTTETETETRRSANYEPNSWDYDYLSSDTDESIEVYKDKAKKLEAEVRRBINNEKAEFTLLELDNVQRLGLGYRFESDIRRALDRFY : 100
          *          *          *          *          *          *          *          *          *          *
IspSm1 : SSGGFDAVTKTSLHATALSFRLLRQHGFEVSQFAFGKQNGNPLENLKEDIKAILSLYASFLAEGENILDEAKVFAISHKELSEEKIGKDLAQV : 200
IspSm2 : SSGGFDAVTKTSLHATALSFRLLRQHGFEVSQFAFGKQNGNPLENLKEDIKAILSLYASFLAEGENILDEAKVFAISHKELSEEKIGKDLAQV : 200
          *          *          *          *          *          *          *          *          *          *
IspSm1 : NHALELPLHRRTORLEAVLSIEAYRKKEADQVLELAILDYNNICSVYQDRLRETSAMWRRVGLATKLHFARDELIESFYWAGVAFEPQYSDCRNSVA : 300
IspSm2 : NHALELPLHRRTORLEAVLSIEAYRKKEADQVLELAILDYNNICSVYQDRLRETSAMWRRVGLATKLHFARDELIESFYWAGVAFEPQYSDCRNSVA : 300
          *          *          *          *          *          *          *          *          *          *
IspSm1 : KMFSPVTIIDDIVYVGTIDLELELFTNAVERMDVNAIDDLPOYMKLCFLALYNTINEIAYDNLKEGENILPYLTKAAGDLCAFLQEAHWLYNKSTPTF : 400
IspSm2 : KMFSPVTIIDDIVYVGTIDLELELFTNAVERMDVNAIDDLPOYMKLCFLALYNTINEIAYDNLKEGENILPYLTKAAGDLCAFLQEAHWLYNKSTPTF : 400
          *          *          *          *          *          *          *          *          *          *
IspSm1 : DDYFGNAWSSSGGFLQVFAVFAVQNIKKEIBNLQKYHDIIIRPSHIFRLCNDLASASAEIARGETANSVSCYMRKGISEELATESVMNLIDETWKK : 500
IspSm2 : DDYFGNAWSSSGGFLQVFAVFAVQNIKKEIBNLQKYHDIIIRPSHIFRLCNDLASASAEIARGETANSVSCYMRKGISEELATESVMNLIDETWKK : 500
          *          *          *          *          *          *          *          *          *          *
IspSm1 : MNKRLGGSLFAKFFVETAINLARQSHCTYHNGDAHTSPDELTRKRVLSVITEPILPFER : 560 (SEQ ID NO: 10)
IspSm2 : MNKRLGGSLFAKFFVETAINLARQSHCTYHNGDAHTSPDELTRKRVLSVITEPILPFER : 560 (SEQ ID NO: 8)
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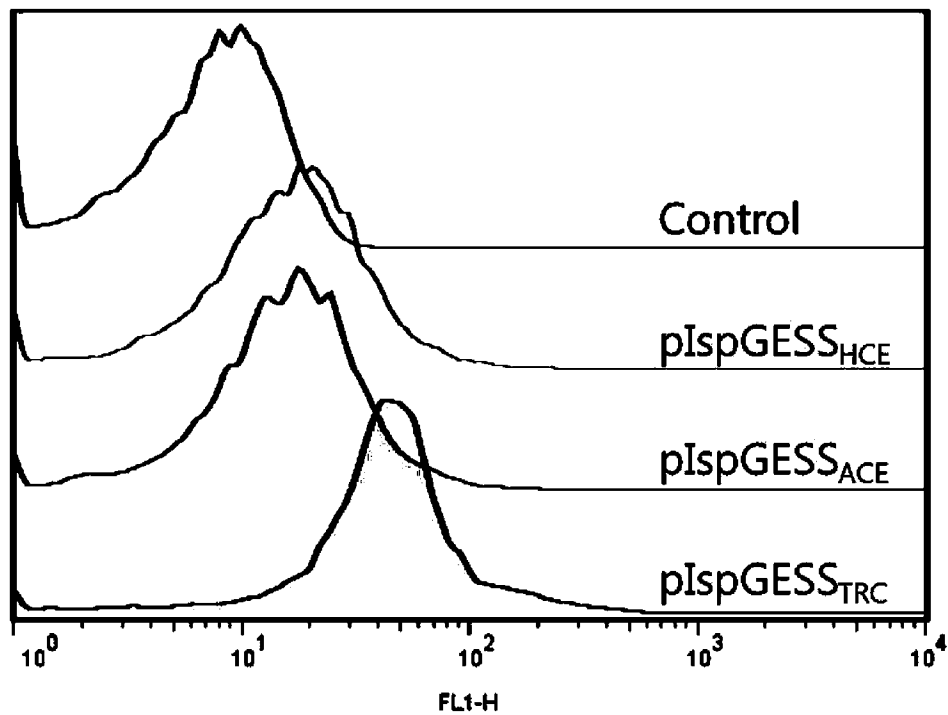
[Fig. 8]



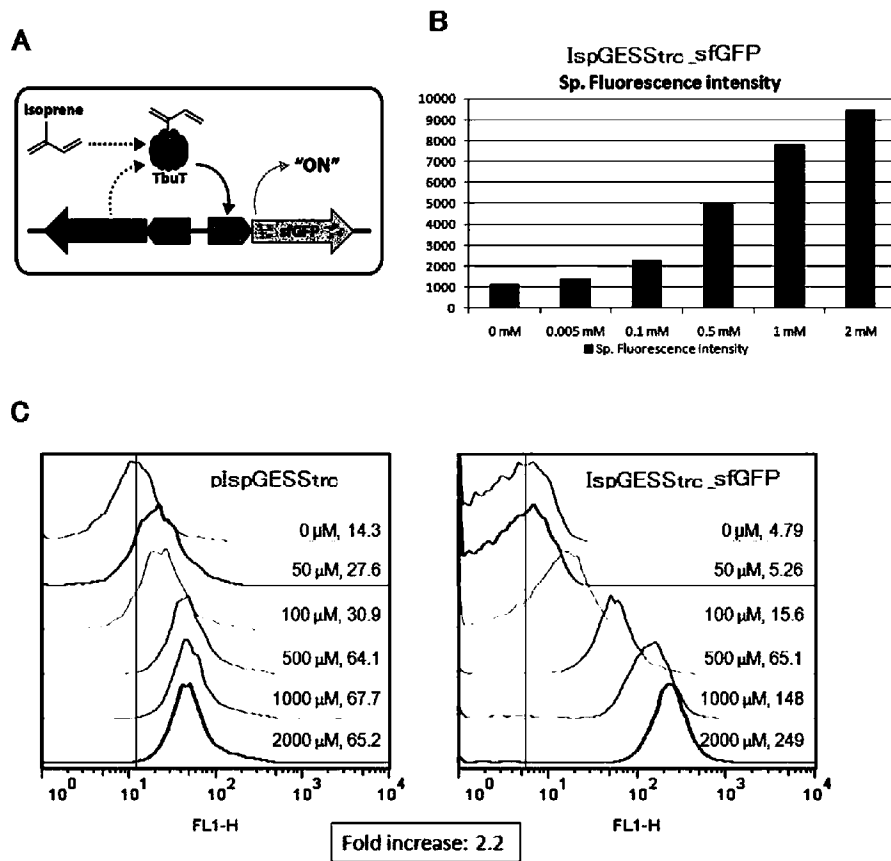
[Fig. 9]



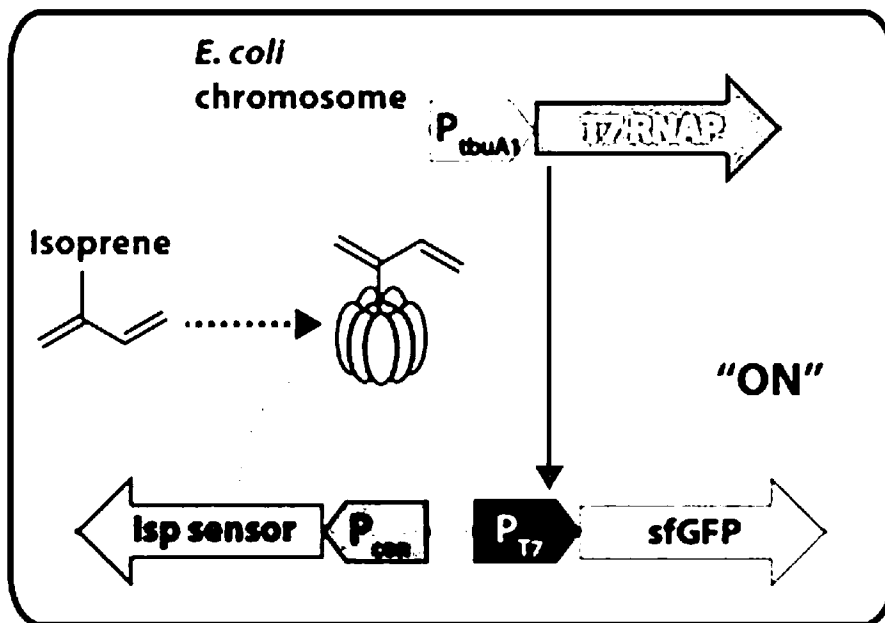
[Fig. 10]



[Fig. 11]

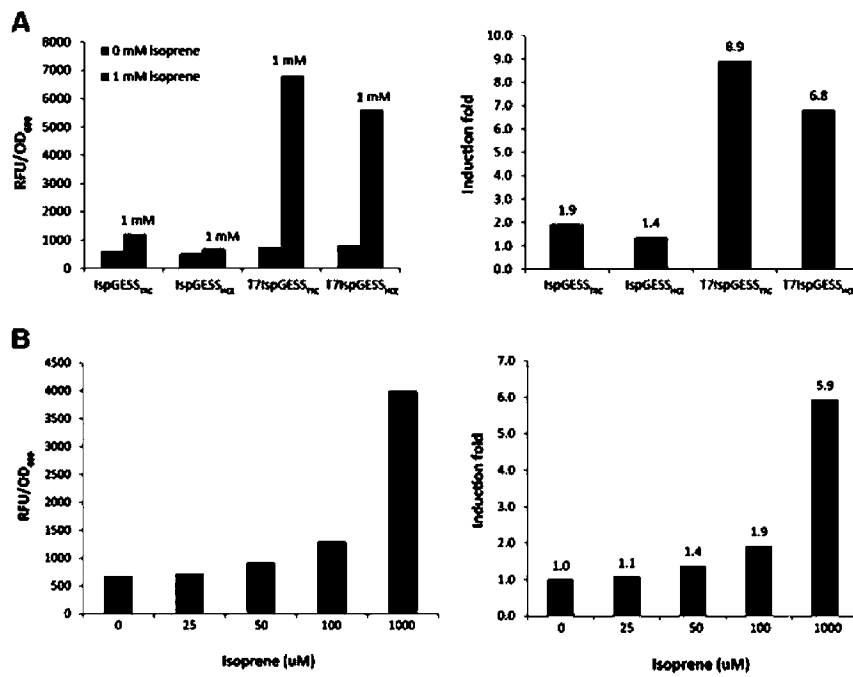


[Fig. 12]

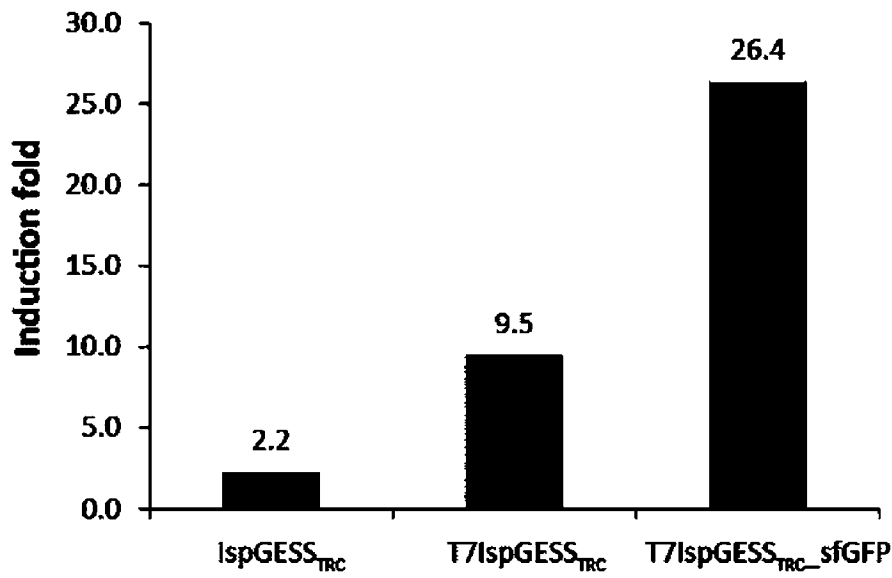


P_{con} : J23105 or J23114

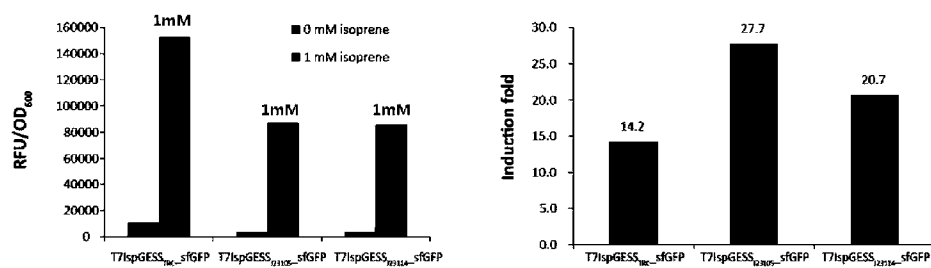
[Fig. 13]



[Fig. 14]



[Fig. 15]



A. CLASSIFICATION OF SUBJECT MATTER**C12Q 1/25(2006.01)i, C12Q 1/68(2006.01)i**

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C12Q 1/25; C40B 30/08; C12Q 1/68

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Korean utility models and applications for utility models

Japanese utility models and applications for utility models

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

eKOMPASS(KIPO internal) & Keywords: screening, quantifying, isoprene, biosynthesis, enzyme activity, artificial genetic circuit, transcriptional regulator, promoter, gene library

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 2012-0238470 A1 (LEE et al.) 20 September 2012 See abstract and claims 1-4.	1-22
A	YANG et al., 'Enhancing production of bio-isoprene using hybrid MVA pathway and isoprene synthase in E. coli' PLoS One, Vol.7, No.4, Article no.e33509 (internal pages 1-7) (27 April 2012) See abstract and page 3.	1-22
A	XUE et al., 'Enhancing isoprene production by genetic modification of the 1-deoxy-d-xylulose-5-phosphate pathway in Bacillus subtilis' Applied and Environmental Microbiology, Vol.77, No.7, pp.2399-2405 (04 February 2011) See abstract and pages 2400-2402.	1-22
A	ZHAO et al., 'Biosynthesis of isoprene in Escherichia coli via methylerythritol phosphate (MEP) pathway' Applied Microbiology and Biotechnology, Vol.90, No.6, pp.1915-1922 (06 April 2011) See abstract and page 1917.	1-22



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

21 May 2014 (21.05.2014)

Date of mailing of the international search report

22 May 2014 (22.05.2014)

Name and mailing address of the ISA/KR

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/KR2014/001360

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	SHARKEY et al., 'Evolution of the isoprene biosynthetic pathway in kudzu' Plant Physiology, Vol.137, No.2, pp.700-712 (February 2005) See abstract and pages 706-708.	1-22
A	NCBI, GenBank, accession no. CAL69918.1 (14 August 2008) See the whole document.	1-22

INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR2014/001360

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of :

a. a sequence listing filed or furnished

- ☐ on paper
☒ in electronic form

b. time of filing or furnishing

- ☒ contained in the international application as filed
☐ filed together with the international application in electronic form
☐ furnished subsequently to this Authority for the purposes of search

2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/KR2014/001360Patent document
cited in search reportPublication
datePatent family
member(s)Publication
date

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