Microbial production of the flavonoids garbanzol, resokaempferol and fisetin

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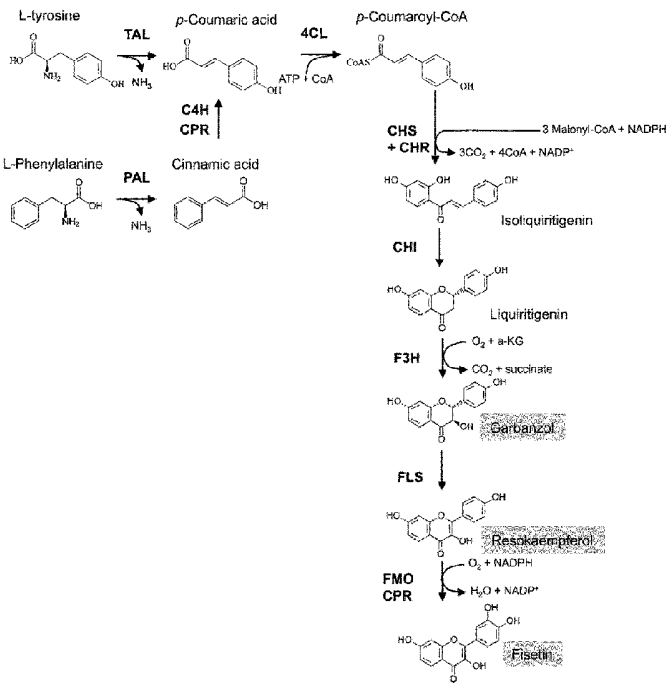
(54) Title: MICROBIAL PRODUCTION OF THE FLAVONOIDS GARBANZOL, RESOKAEMPFEROL AND FISETIN

Figure 1



(57) Abstract: The invention provides a genetically modified micro-organism comprising one or more transgene for the production of one or more of the flavonoids garbanzol, resokaempferol and fisetin. The micro-organism may be a bacterial or yeast cell engineered to express a metabolic pathway for garbanzol, resokaempferol and/or fisetin biosynthesis. The invention further provides a method for producing garbanzol, resokaempferol and/or fisetin employing the genetically modified micro-organism of the invention. The genetically modified micro- organism may be used to convert a number of substrates and/or co-substrates into fisetin via a fisetin biosynthetic pathway.

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TITLE Microbial Production of the flavonoids garbanzol, resokaempferol and fisetin

Field of the invention

The invention provides genetically modified micro-organisms comprising one or more transgene for the production of one or more of the flavonoids garbanzol, resokaempferol and fisetin. The micro-organisms may be a bacterial, yeast or filamentous fungus cells engineered to express a metabolic pathway for garbanzol and/or resokaempferol and/or fisetin biosynthesis. The invention further provides a method for producing garbanzol, resokaempferol and fisetin employing a genetically modified micro-organism of the invention. The genetically modified micro-organism is capable of using a number of substrates and/or co-substrates for garbanzol, resokaempferol and fisetin synthesis including L-tyrosine, L-phenylalanine, glucose, maltose, fructose, sucrose, arabinose, xylose, raffinose, mannose, lactose, erythrose, threose, ribose, ethanol, acetate, glycerol, lactate, p-coumaric acid, liquiritigenin, garbanzol, and resokaempferol.

Background of the Invention

Flavonoids are highly ubiquitous polyphenolic secondary metabolites produced in many plants. Based on molecular structure, they can be subdivided into flavonols, flavones, flavonones, isoflavones, catechins, and anthocyanins. These plant secondary metabolites are an important source of new drugs and nutraceuticals. Polyphenols have been shown to have anti-inflammatory, anti-oxidant, anti-viral, anti-bacterial, anti-cancer as well as anti-proliferative, and anti-arteriosclerotic activities.

Fisetin is a bioactive flavonol molecule found in fruits and vegetables such as strawberry, apple, persimmon, grape, onion, and cucumber at concentrations ranging from 2 to 160 µg/g (Khan et al., 2012). Fisetin has been reported to display anti-aging (Maher et al., 2009), anti-inflammatory (Cho, et al., 2012; Kim et al., 2013), anti-carcinogenic (Ying et al., 2012) and anti-viral (Kang et al., 2012; Zandi et al., 2011) properties. In addition, it is claimed to be an orally active neuroprotective and memory-enhancing molecule (Maher et al., 2006; Sagara et al., 2004). Recently, fisetin has been suggested as a new approach for the treatment of Alzheimer's disease (Currais et al., 2013). Additionally, fisetin was shown to prevent complications associated with diabetes type I (Maher et al., 2011). Furthermore, other flavonoid compounds, such as the flavonols garbanzol and resokaempferol have been associated with health beneficial effects against diabetes and cancer (Fowler and Koffas, 2009).

Today, many flavonoids are obtained by either time consuming and laborious plant extractions in which the efficient production of pure flavonoid compounds continues to be a

major challenge for drug development; or by chemical synthesis, where toxic chemicals, extreme reaction conditions and complexity of the flavonoid itself limits the *de novo* synthesis of these compounds (Matkowski, A. 2008; Keasling., 2010).

Miyahisa et al., 2006 describes recombinant *Escherichia coli* cells containing genes encoding PAL, CCL (4CL), CHS, CHI, as well as F3H and FLS from Citrus species leading to the synthesis of kaempferol from tyrosine. The intermediates in this pathway leading to the synthesis of kaempferol are cinnamoyl-CoA; naringenin chalcone, naringenin, and dihydrokaempferol. However, the flavonoids garbanzol, resokaempferol and fisetin are neither intermediates nor products of this pathway.

Microbial production of flavonoids has emerged as an excellent economical alternative, using a production process characterized by simple, readily available, inexpensive starting materials, as well as low waste emission and energy requirements. Therefore, assembly of biosynthetic pathways into microbes followed by metabolic engineering of the resulting recombinant microbial strain appears to be a promising alternative for the large-scale production of flavonoids. In view of these advantages, it would be desirable to use micro-organisms for the production of the flavonoids garbanzol, resokaempferol and fisetin. However, despite the fact each of these flavonoids are known to exist in plants, their mode of synthesis *in vivo* remains unknown. Thus there exists a need for constructing a metabolic pathway for the biosynthesis of garbanzol, resokaempferol and fisetin, and genetically engineering a micro-organism to carry out the pathway for garbanzol and/or resokaempferol and/or fisetin synthesis *in vivo*.

Summary of the invention

The invention provides a genetically modified micro-organism for production of fisetin; wherein said microorganism comprises one or more transgene encoding one or more polypeptide; wherein the more or more polypeptide have an enzymatic activity of a flavanone 3-hydroxylase; a flavonol synthase; and a flavonoid 3'-monooxygenase; wherein one of said transgene or a native gene of said micro-organism encodes a polypeptide having the enzymatic activity of a cytochrome P450 reductase.

In a further embodiment the genetically modified micro-organism for production of fisetin may additionally comprise one or more transgene encoding one or more polypeptide having an enzymatic activity selected from among (a) a chalcone isomerase;(b), a chalcone synthase, a chalcone reductase and a chalcone isomerase; (c) a 4-coumaroyl-CoA ligase, a chalcone synthase, a chalcone reductase and a chalcone isomerase; (d) tyrosine ammonia-lyase, a 4-coumaroyl-CoA ligase, chalcone synthase, a chalcone reductase and a chalcone

isomerase; (e) a phenylalanine ammonia lyase, a 4-coumaroyl-CoA ligase, chalcone synthase, a chalcone reductase and a chalcone isomerase and (f) a phenylalanine ammonia lyase, a cinnamate 4-hydroxylase and a NADPH:cytochrome P450 reductase, a 4-coumaroyl-CoA ligase, chalcone synthase, a chalcone reductase and a chalcone isomerase. Optionally, the above genetically modified micro-organism comprises a transgene encoding a fusion polypeptide wherein one domain of the fused polypeptide has chalcone synthase activity and the adjoining domain has chalcone reductase acitivity.

The genetically modified micro-organism is preferably selected from among a bacterium, yeast, and a filamentous fungus.

In one embodiment of the genetically modified micro-organism, the polypeptide having flavanone 3-hydroxylase (F3H) activity comprises an amino acid sequence having at least 80% sequence identity to the amino acid sequence of any one of Arabidopsis thaliana F3H (SEQ ID NO: 2); *Capsella rubella* F3H (SEQ ID NO: 4); *Brassica napus* F3H (SEQ ID NO: 6); *Eutrema salsugineum* F3H (SEQ ID NO: 8); *Gossypium hirsutum* F3H (SEQ ID NO: 10); *Ampelopsis grossedentata* F3H (SEQ ID NO: 12); *Theobroma cacao* F3H (SEQ ID NO: 14); and *Petunia x hybrida* F3H (SEQ ID NO: 16). Preferably, the the polypeptide having flavanone 3-hydroxylase (F3H) activity comprises the amino acid sequence having at least 90% amino acid sequence identity to any one of SEQ ID No. 2, 4, 6, and 8.

In one embodiment of the genetically modified micro-organism the polypeptide having flavonol synthase (FLS) activity comprises an amino acid sequence having at least 80% sequence identity to the amino acid sequence of any one of *Arabidopsis thaliana* FLSat (SEQ ID NO: 18); *Arabidopsis lyrata* FLSal (SEQ ID NO: 20); *Capsella rubella* FLScr (SEQ ID NO: 22); *Eutrema salsugineum* FLS (SEQ ID NO: 24); *Morus notabilis* FLS (SEQ ID NO: 26); *Citrus unshiu* FLS (SEQ ID NO: 28); *Theobroma cacao* FLS (SEQ ID NO: 30); *Vitis vinifera* FLS (SEQ ID NO: 32); and *Eucalyptus grandis* FLS (SEQ ID NO: 34). Preferably the polypeptide having flavonol synthase (FLS) activity comprises an amino acid sequence at least 90% amino acid sequence identity to any one of SEQ ID NOs: 18, 20, 22 and 24.

In one embodiment of the genetically modified micro-organism the polypeptide having flavonoid 3'-monooxygenase (FMO) activity comprises an amino acid sequence having at least 80% sequence identity to the amino acid sequence of any one of *Arabidopsis thaliana* FMO (SEQ ID NO: 36); two subspecies of Fragaria x ananassa FMO 1 and 2 (SEQ ID No:40 and SEQ ID No: 42); *Petunia hybrida* FMO (SEQ ID NO: 38); and *Malus x domestica* FMO (SEQ ID No. 44); *Eutrema salsugineum* (SEQ ID NO: 46); *Capsella rubella* (SEQ ID NO: 48); *Nicotiana tabacum* (SEQ ID NO: 50); and *Vitis vinifera* (SEQ ID NO: 52). Preferably the polypeptide having flavonoid 3'-monooxygenase (FMO) activity has at least 90% amino acid

sequence identity to any one of SEQ ID NOs: 36, 46 and 48; or to any one of SEQ ID No: 36, 38 and 42.

In one embodiment of the genetically modified micro-organism, the polypeptide having cytochrome P450 reductase (CPR) activity comprises an amino acid sequence having at least 80% sequence identity to the amino acid sequence of any one of *Catharanthus roseus* CPR (SEQ ID No: 54); *Arabidopsis thaliana* CPR (1) (SEQ ID NO: 56) or CPR (2) (SEQ ID NO: 58); *Petunia x hybrid* CPR (1) (SEQ ID NO: 60) and CPR (2) (SEQ ID NO: 62); *Vitis vinifera* CPR (SEQ ID NO: 64); and *Capsella rubella* CPR (SEQ ID NO: 66). Preferably the polypeptide having cytochrome P450 reductase (CPR) activity has at least 90% amino acid sequence identity to any one of SEQ ID NOs: 54, 60, and 64.

In one embodiment of the genetically modified micro-organism, the polypeptide having tyrosine ammonia-lyase (TAL) activity comprises an amino acid sequence having at least 80% sequence identity to the amino acid sequence of any one of *Rhodobacter sphaeroides* TAL (SEQ ID NO: 68); *Flavobacterium johnsoniae* TAL (SEQ ID NO: 70); *Saccharothrix espanaensis* TAL (SEQ ID NO: 72); *Herpetosiphon aurantiacus* TAL (SEQ ID NO: 74) and (SEQ ID NO: 76); *Rhodotorula mucilaginosa / Rhodotorula rubra* TAL (SEQ ID NO: 78); and *Streptomyces sp. Tu 4128* TAL (SEQ ID NO: 80). Preferably, the polypeptide having tyrosine ammonia-lyase (TAL) activity has at least 90% sequence identity to any one of the SEQ ID NOs: 70, 74 and 76; more preferably SEQ ID NOs: 74 and 76.

In one embodiment of the genetically modified micro-organism, the polypeptide having 4-coumaroyl-CoA lyase (4CL) activity comprises an amino acid sequence having at least 80% sequence identity to the amino acid sequence of any one of *Petroselinum crispum* 4CL (SEQ ID NO: 82); *Lithospermum erythrorhizon* (SEQ ID NO: 84); *Streptomyces coelicolor* (SEQ ID NO: 86); *Paulownia fortunei* (SEQ ID NO: 88); *Fraxinus mandshurica* (SEQ ID NO: 90); *Nicotiana tabacum* (SEQ ID NO: 92); *Streptomyces violaceoruber* (SEQ ID NO: 94); Streptomyces coelicoflavus ZG0656 (SEQ ID NO: 96); and *Streptomyces violaceorubidus* (SEQ ID NO: 98). Preferably, the polypeptide having 4-coumaroyl-CoA lyase (4CL) activity has at least 92% sequence identity to any one of SEQ ID NOs: 82, 84, 88, 90 and 92.

In one embodiment of the genetically modified micro-organism, wherein the polypeptide having chalcone synthase (CHS) activity comprises an amino acid sequence having at least 80% sequence identity to the amino acid sequence of any one of of *Arabidopsis thaliana* CHS (SEQ ID No: 102); *Petunia x hybrid* CHS (SEQ ID No: 100); *Fragaria x ananassa* CHS (SEQ ID No: 104); *Malus domestica* CHS (SEQ ID No: 106); *Vitis vinifer*a CHS (SEQ ID No: 108); *Hypericum androsaemum* CHS (SEQ ID No: 110); and *Nicotiana alata* CHS (SEQ ID

No: 112). Preferably, the polypeptide having 4- chalcone synthase (CHS) activity has at least 90% sequence identity to any one of SEQ ID NOs: 100, 104, 106, 108, 110, and 112.

In one embodiment of the genetically modified micro-organism, wherein the polypeptide having chalcone reductase (CHR) activity comprises an amino acid sequence having at least 80% sequence identity to the amino acid sequence of any one of *Medicago sativa* CHR (SEQ ID No: 114); *Arabidopsis thaliana* CHR (SEQ ID No: 116); *Fragaria x ananassa* CHR (SEQ ID No: 118) and (SEQ ID No: 120).; *Malus domestica* CHR (SEQ ID No: 122); *Vitis vinifera* CHR (SEQ ID No: 126); *Astragalus mongholicus* CHR (SEQ ID No: 128); *Malus domestica* CHR (SEQ ID No: 124); and *Sesbania rostrata* CHR (SEQ ID No: 130). Preferably, the polypeptide having chalcone reductase (CHR) activity has at least 85% amino acid sequence identity to any one of SEQ ID NOs: 114 and 128

In one embodiment of the genetically modified micro-organism, wherein the polypeptide having chalcone isomerase (CHI) activity comprises an amino acid sequence having at least 80% sequence identity to the amino acid sequence of any one of *Medicago sativa* CHI (SEQ ID No: 132) and (SEQ ID No: 134).; *Lotus japonicus* CHI (SEQ ID No: 136); *Medicago truncatula* CHI (SEQ ID No: 138); *Glycine max* CHI (SEQ ID No: 140); *Glycyrrhiza uralensis* CHI (SEQ ID No: 142); *Pueraria montana* var. *lobata* CHI (SEQ ID No: 144); *Phaseolus vulgaris* CHI (SEQ ID No: 146); *Astragalus mongholicus* CHI (SEQ ID No: 148). Preferably, the polypeptide having chalcone isomerase (CHI) activity has at least 80% amino acid sequence identity to any one of SEQ ID NOs: 132, 138, 140, 142 and 146.

In one embodiment of the genetically modified micro-organism, wherein the polypeptide having cinnamate 4-hydroxylase comprises an amino acid sequence having at least 80% sequence identity to the amino acid sequence of A. thaliana C4H (SEQ ID No: 152) and NADPH:cytochrome P450 reductase activity comprises an amino acid sequence having at least 80% sequence identity to the amino acid sequence of *A. thaliana* CPR (SEQ ID No: 58) and *Saccharomyces cerevisiae* CYP5 (SEQ ID No. 154). Additionally, where the genetically modified micro-organism comprises a polypeptide having cinnamate 4-hydroxylase, preferably the polypeptide has at least 80% amino acid sequence identity to SEQ ID NOs: 152.

In one embodiment, the genetically modified micro-organism, comprises one or more transgene encoding one or more polypeptide; wherein the more or more polypeptide has an enzyme activity of:

- a tyrosine ammonia-lyase having an amino acid sequence having at least 90% identity to any one of SEQ ID NOs: 70, 74 and 76; preferably at least 90% identity to SEQ ID NOs: 74 and 76; and

- a 4-coumaroyl-CoA ligase having an amino acid sequence having at least 79% identity to any one of SEQ ID NOs: 82, 88, and 92 (for example SEQ ID No. 82); and
- a chalcone synthase having an amino acid sequence having at least 90% identity to any one of SEQ ID NOs:100, 104, 106, 108, 110, and 112 (for example SEQ ID No. 100); and
- a chalcone reductase having an amino acid sequence having at least 88% identity to any one of SEQ ID NOs: 114 and 128, and
- a chalcone isomerase having an amino acid sequence having at least 80% identity to any one of SEQ ID NOs: 132, 138, 140, 142, and 146 (or for example one of SEQ ID No. 144, 146 and 150); and
- a flavanone 3-hydroxylase having an amino acid sequence having at least 90% identity to any one of SEQ ID NOs:2, 4, 6, and 8 (for example SEQ ID No. 2); and
- a flavonol synthase having an amino acid sequence having at least 90% identity to any one of SEQ ID NOs: 18, 20, 22 and 24 (for example SEQ ID No. 18); and
a flavonoid 3'-monooxygenase having an amino acid sequence having at least 90% identity to any one of SEQ ID NOs: 36, 38, 46, and 48 (for example SEQ ID No. 36 or 38); and
- a cytochrome P450 reductase having an amino acid sequence having at least 79% identity to SEQ ID NOs: 54.

In respect of this immediately previous embodiment of the genetically modified micro-organism; the polypeptide having tyrosine ammonia-lyase activity may be substituted by one or more polypeptide having the activity of:
- a phenylalanine ammonia lyase having an amino acid sequence having at least 90% identity SEQ ID NOs:150; and
- a cinnamate 4-hydroxylase having an amino acid sequence having at least 90% identity to SEQ ID NOs:152 or and
- a NADPH:cytochrome P450 reductase having an amino acid sequence having at least 90% identity to SEQ ID NOs: 58 or 154.

In a further embodiment, the genetically modified micro-organism is a yeast cell for the production of fisetin from L- tyrosine and/or L-phenylalanine as described herein, wherein the yeast further comprises a mutant ARO4 gene encoding a 3-deoxy-D-arabino-heptulosonate-7- phosphate synthase having a K229L substitution; and a mutant ARO7 gene encoding chorismate mutase having a G141S substitution. Over-expression of both the feedback-inhibition resistant allele of 3-deoxy-D-arabino-heptulosonate-7- phosphate synthase ARO4K229L and the feedback-inhibition resistant allele of chorismate mutase ARO7G141S from *S. cerevisiae* is found to improve the biosynthesis of tyrosine and phenylalanine.

In a further embodiment, the invention provides a method for the production of fisetin, comprising the steps of: a) introducing a genetically modified microorganism for the production of fisetin into a growth medium to produce a culture; b) providing a substrate for the production of fisetin; and c) recovering fisetin produced by said culture.

In one embodiment of the method for the production of fisetin, the growth medium comprises one or more substrate and or co-substrate for production of fisetin selected from among liquiritigenin, L-tyrosine, glucose, maltose, fructose, sucrose, arabinose, xylose, raffinose, mannose, lactose, erythrose, threose, ribose, ethanol, acetate, glycerol, lactate.

In a further embodiment, the invention describes the use of a genetically modified micro-organism for production of fisetin; wherein the micro-organism comprises one or more transgene encoding one or more polypeptide, wherein the more or more polypeptide have an enzymatic activity of (a) tyrosine ammonia-lyase, a 4-coumaroyl-CoA ligase, a chalcone synthase, a chalcone reductase, a chalcone isomerase, a flavanone 3-hydroxylase, a flavonol synthase, and a flavonoid monooxygenase (either EC 1.14.13.21 or EC:1.14.13.88); or (b) a phenylalanine ammonia lyase, a cinnamate 4-hydroxylase, a 4-coumaroyl-CoA ligase, chalcone synthase, a chalcone reductase, a chalcone isomerase, a flavanone 3-hydroxylase, a flavonol synthase, and a flavonoid monooxygenase (either EC 1.14.13.21 or EC:1.14.13.88); and wherein one of said transgene or a native gene of said micro-organism encodes a polypeptide having the enzymatic activity of a cytochrome P450 reductase. Optionally, the above genetically modified micro-organism comprises a transgene encoding a fusion polypeptide wherein one domain of the fused polypeptide has chalcone synthase activity and the adjoining domain has chalcone reductase acitivity.

In a further embodiment, the invention provides a genetically modified micro-organism for production of garbanozol; wherein said microorganism comprises one or more transgene encoding one or more polypeptide; wherein the more or more polypeptide have an enzymatic activity of a tyrosine ammonia-lyase, a 4-coumaroyl-CoA ligase, a chalcone synthase, a chalcone reductase, a chalcone isomerase and a flavanone 3-hydroxylase, where the transgenes encode one or more polypeptide having a sequence described herein. Optionally, the above genetically modified micro-organism comprises a transgene encoding a fusion polypeptide wherein one domain of the fused polypeptide has chalcone synthase activity and the adjoining domain has chalcone reductase acitivity.

In a further embodiment, the invention provides a genetically modified micro-organism for production of garbanozol, wherein said microorganism comprises one or more transgene encoding one or more polypeptide; wherein the more or more polypeptide have an enzymatic activity of a phenylalanine ammonia lyase, a 4-coumaroyl-CoA ligase, a chalcone

synthase, a chalcone reductase, a chalcone isomerase, and a flavanone 3-hydroxylase, and wherein at least one of said transgene or a native gene of said micro-organism encodes a polypeptide having the enzymatic activity of a cinnamate 4-hydroxylase and a NADPH:cytochrome P450 reductase, and where the transgenes encode one or more a polypeptide having a sequence described herein. Optionally, the above genetically modified micro-organism comprises a transgene encoding a fusion polypeptide wherein one domain of the fused polypeptide has chalcone synthase activity and the adjoining domain has chalcone reductase acitivity.

In a further embodiment, the invention provides a genetically modified micro-organism for production of resokaempferol; wherein said microorganism comprises one or more transgene encoding one or more polypeptide; wherein the more or more polypeptide have an enzymatic activity of a tyrosine ammonia-lyase, a 4-coumaroyl-CoA ligase, a chalcone synthase, a chalcone reductase, a chalcone isomerase, a flavanone 3-hydroxylase and a flavonol synthase, where the transgenes encode one or more polypeptide having an amino acid sequence described herein. Optionally, the above genetically modified micro-organism comprises a transgene encoding a fusion polypeptide wherein one domain of the fused polypeptide has chalcone synthase activity and the adjoining domain has chalcone reductase acitivity.

In a further embodiment, the invention provides a genetically modified micro-organism for production of resokaempferol, wherein said microorganism comprises one or more transgene encoding one or more polypeptide; wherein the more or more polypeptide have an enzymatic activity of a phenylalanine ammonia lyase, a 4-coumaroyl-CoA ligase, a chalcone synthase, a chalcone reductase, a chalcone isomerase, a flavanone 3-hydroxylase, and a flavonol synthase, wherein at least one of said transgene or a native gene of said micro-organism encodes a polypeptide having the enzymatic activity of a cinnamate 4-hydroxylase and a NADPH:cytochrome P450 reductase, where the transgenes encode one or more a polypeptide having an amino acid sequence described herein. Optionally, the above genetically modified micro-organism comprises a transgene encoding a fusion polypeptide wherein one domain of the fused polypeptide has chalcone synthase activity and the adjoining domain has chalcone reductase acitivity.

The genetically modified micro-organism for production of garbanzol and/or resokaempferol, is preferably selected from among a bacterium, a yeast, and a filamentous fungus.

In a further embodiment, the invention provides a method for the production of garbanzol and/or resokaempferol, comprising the steps of: a) introducing a genetically modified microorganism the production of garbanzol and/or resokaempferol into a growth medium to

produce a culture; b) providing a substrate for the production of garbanzol and/or resokaempferol; and c) recovering garbanzol and/or resokaempferol produced by said culture.

In a further embodiment, the invention describes the use of a genetically modified micro-organism for production of garbanzol and/or resokaempferol.

Description of the invention

FIGURES

Figure 1. Novel microbial pathway for the synthesis of the flavonoid fisetin. PAL, phenylalanine ammonia-lyase; C4H, Cinnamate 4-hydrolase (P450); CPR, cytochrome P450 reductase; TAL, tyrosine ammonia-lyase; 4CL, 4-coumaroyl-CoA lyase; CHS, chalcone synthase; CHR, chalcone reductase; CHI, chalcone isomerase; F3H, Flavanone 3-hydroxylase; FLS, Flavonol synthase and FMO, flavonoid 3'-monooxygenase; and CPR, cytochrome P450 reductase.

Figure 2. Typical HPLC profile at 333nm of flavonoid compounds extractable from (A) Liquiritigenin-supplemented *E. coli* strain ST3 culture; (B) Liquiritigenin-supplemented *E. coli* strain carrying pCDFDuet-1 culture. (C) UV spectra of compounds analysed by HPLC: ILQ – Isoliquiritigenin, LIQ – liquiritigenin, GAR – garbanzol and RSK - resokaempferol. Experiments were carried out in triplicates.

Figure 3. The spectra of infused isolated peaks and standards in a group of four; the positive ms1 spectrum is top left, the positive ms2 spectrum is top right, the negative ms1 spectrum is bottom left and the negative ms2 spectrum is bottom right for A) Purified garbanzol, B) Purified resokaempferol and C) Standard of resokaempferol.

Figure 4. Concentration of fisetin extractable from samples of A) resokaempferol-supplemented cultures of *E. coli* strains carrying truncated codon optimized CPR from *C. roseus* and codon optimized FMO from *A. thaliana* (ST4at), *P. hybrida* (ST4ph), as well as F3'5'H from *C. roseus* (ST4cr), and an empty plasmid negative control (pACYC) taken after 3hours (white bars), 18hours (grey bars) and 40hours (black bars) after addition of resokaempferol; and B) Liquiritigenin-supplemented cultures of *E. coli* strains carrying F3H and FLS from *A. thaliana* and codon optimized FMO from A. thaliana (ST5at) or *P. hybrida* (ST5ph), and an empty plasmid negative control (pACYC) after 3 hours (white bars), 6 hours (light grey bars) and 18 hours (grey bars) after addition of resokaempferol; and C)

Liquiritigenin-supplemented cultures of *E. coli* strains carrying F3H and FLS from *A. thaliana* and codon optimized FMO from *A. thaliana* (ST5at) or *P. hybrida* (ST5ph) and an empty plasmid negative control (pACYC) after 24 hours. Experiments were carried out in triplicates. The mean values of three experiments are shown along with standard error of mean. P values are calculated by Student's t-test, with welch corrections.

Figure 5. The spectra of infused isolated peaks and standards in a group of four; the positive ms1 spectrum is top left, the positive ms2 spectrum is top right, the negative ms1 spectrum is bottom left and the negative ms2 spectrum is bottom right for A) The isolated peak identified as fisetin, and B) The standard of fisetin.

Figure 6. The figure shows on the left 4 pairs of chromatogram, each pair are the total ion chromatogram (Top) and the extracted ion chromatogram (XIC) for the m/z that corresponds to the protonated molecular ion. The pairs of chromatograms are for the following samples from top to bottom, fisetin standard, sample extract from ST_NEW6, sample extract from ST_NEW5 and a negative control. Fisetin can be observed in the authentic standard, the ST_NEW6 and slightly in ST_NEW5. Similarly the spectra corresponding to the retention time of the peak in the authentic standard (shown in the right panel) show evidence for an ion corresponding to the correct m/z value for that observed for fisetin in the ST_NEW6 and slightly in ST_NEW5.

Abbreviations and terms:

gi number: (genInfo identifier) is a unique integer which identifies a particular sequence, independent of the database source, which is assigned by NCBI to all sequences processed into Entrez, including nucleotide sequences from DDBJ/EMBL/GenBank, protein sequences from SWISS-PROT, PIR and many others.

Amino acid sequence identity: The term "sequence identity" as used herein, indicates a quantitative measure of the degree of homology between two amino acid sequences of substantially equal length. The two sequences to be compared must be aligned to give a best possible fit, by means of the insertion of gaps or alternatively, truncation at the ends of the protein sequences. The sequence identity can be calculated as ((Nref-Ndif)100)/(Nref), wherein Ndif is the total number of non-identical residues in the two sequences when aligned and wherein Nref is the number of residues in one of the sequences. Sequence identity can alternatively be calculated by the BLAST program e.g. the BLASTP program (Pearson W.R and D.J. Lipman (1988)) (www.ncbi.nlm.nih.gov/cgi-bin/BLAST). In one embodiment of the invention, alignment is performed with the sequence alignment method

ClustalW with default parameters as described by Thompson J., et al 1994, available at http://www2.ebi.ac.uk/clustalw/.

Preferably, the numbers of substitutions, insertions, additions or deletions of one or more amino acid residues in the polypeptide as compared to its comparator polypeptide is limited, i.e. no more than 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 substitutions, no more than 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 insertions, no more than 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 additions, and no more than 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 deletions. Preferably the substitutions are conservative amino acid substitutions: limited to exchanges within members of group 1: Glycine, Alanine, Valine, Leucine, Isoleucine; group 2: Serine, Cysteine, Selenocysteine, Threonine, Methionine; group 3: proline; group 4: Phenylalanine, Tyrosine, Tryptophan; Group 5: Aspartate, Glutamate, Asparagine, Glutamine.

Native gene: endogenous gene in a microbial cell genome, homologous to host micro-organism.

Detailed description of the invention

The present invention provides a novel biosynthetic pathway for the synthesis of garbanzol, resokaempferol, and/or fisetin, starting from a range of substrates including carbohydrates, aromatic amino acids, liquiritigenin or other pathway intermediates. Synthesis of one or more of garbanzol, resokaempferol and fisetin, is demonstrated in a micro-organism genetically modified to carry out this novel pathway.

I A micro-organism for production of fisetin from liquiritigenin

The invention provides a genetically modified micro-organism comprising genes whose expression provides a novel metabolic pathway for the synthesis of fisetin. In a first embodiment, the pathway comprises three steps that convert liquiritigenin into fisetin through garbanzol and resokaempferol catalyzed by the enzymes flavanone 3-hydroxylase (F3H); flavonol synthase (FLS); and finally flavonoid 3'-monooxygenase (FMO) and cytochrome P450 reductase (CPR) utilizing O_2, NADPH and alpha ketoglutarate as cofactors (figure 1).

Accordingly, the micro-organism of the invention is characterized by having one or more genes encoding four enzymatic activities, F3H (EC 1.14.11.9); FLS (EC 1.14.11.23); FMO (EC 1.14.13.21), and CPR (EC 1.6.2.4). One or more of the four enzymatic activities and their corresponding enzymatic domains, may be present in individual proteins, each encoded by a gene, or one or more of the enzymatic activities may be present in fusion proteins, where the fusion protein comprises more than one active enzymatic domain encoded by a gene. The genes in the micro-organism of the invention that express a

polypeptide comprising one or more active enzymatic domain, may be transgenes that are adapted for expression in the selected host cell, by employing a codon usage optimized for the given host cell, such codon optimization being well-known in the art. In one embodiment, the genetically modified micro-organism expresses a cytochrome P450 reductase (CPR) encoded by a native gene present in the micro-organism's genome, for example in the yeast genome. Nucleic acid molecules encoding a polypeptide having one or more enzymatic activities can be synthesized chemically, where the nucleic acid sequence of the molecule is selected to provide the codon usage optimized for the given host cell.

Methods for introducing one or more transgene encoding the encoding polypeptides having one or more of the four enzymatic activities into a host micro-organism of the invention is described in section V.

The synthesis of fisetin in cells of the micro-organism of the invention expressing the four enzymatic activities (F3H, FLS, FMO and CPR) and a substrate or co-substrate selected from liquiritigenin, garbanzol and resokaempferol is demonstrated using both bacterial cells (Examples 9 - 11) and in fungal cells from tyrosine (Example 12).

Ii Transgenic expression of a flavanone 3-hydroxylase

A polypeptide having flavanone 3-hydroxylase (F3H) activity, that converts liquiritigenin to garbanzol, comprises an amino acid sequence having at least 70% sequence identity to the amino acid sequence of of *Arabidopsis thaliana* F3H (gi 15230433; SEQ ID NO: 2). Alternatively, the F3H polypeptide may be selected from among *Arabidopsis thaliana* F3H (gi 15230433; SEQ ID NO: 2); *Capsella rubella* F3H (gi 565469330; SEQ ID NO: 4); *Brassica napus* F3H (gi 82795264; SEQ ID NO: 6); *Eutrema salsugineum* F3H (gi 567187905; SEQ ID NO: 8); *Gossypium hirsutum* F3H (gi 121755803; SEQ ID NO: 10); *Ampelopsis grossedentata* F3H (gi 395760140; SEQ ID NO: 12); *Theobroma cacao* F3H (gi 590702748; SEQ ID NO: 14); and *Petunia x hybrida* F3H (gi 2465434; SEQ ID NO: 16).

In another embodiment the polypeptide having F3H activity may have at least 75, 80, 85, 90, 92, 95, 98 or 100% amino acid sequence identity to any one of SEQ ID NOs: 2, 4, 6, 8, 10, 12, 14 and 16; more preferably at least 90% amino acid sequence identity to any one of SEQ ID NOs: 2, 4, 6 and 8 for F3Hat; F3Hcr; F3Hbn; F3Hes respectively.

Iii Transgenic expression of a flavonol synthase

A polypeptide having flavonol synthase (FLS) activity, that converts garbanzol to resokaempferol, comprises an amino acid sequence having at least 70% sequence identity

to the amino acid sequence of *Arabidopsis thaliana* FLS (gi 334187530; SEQ ID NO: 18). Alternatively, the FLS polypeptide may be selected from among *Arabidopsis thaliana* FLS (gi 334187530; SEQ ID NO: 18); *Arabidopsis lyrata* FLS (gi 169635724; SEQ ID NO: 20); *Capsella rubella* FLS (gi 565460290; SEQ ID NO: 22); *Eutrema salsugineum* FLS (gi 567171866; SEQ ID NO: 24); *Morus notabilis* FLS (gi 587874972; SEQ ID NO: 26); *Citrus unshiu* FLS (gi 14916566; SEQ ID NO: 28); *Theobroma cacao* FLS (gi 590637878; SEQ ID NO: 30); *Vitis vinifera* FLS (gi 84794468; SEQ ID NO: 32); and *Eucalyptus grandis* FLS (gi 629078578; SEQ ID NO: 34).

The polypeptide having FLS activity may have at least 75, 80, 85, 90, 92, 94, 98 or 100% sequence identity to any one of SEQ ID NOs: 18, 20, 22, 24, 26, 28, 30, 32 and 34; more preferably at least 90% amino acid sequence identity to any one of SEQ ID NOs: 18, 20, 22 and 24 for FLSat; FLSAl, FLScr and FLSes respectively.

Iiii Transgenic expression of a flavonoid 3'-monooxygenase and cytochrome P450 reductase

A polypeptide having flavonoid 3'-monooxygenase (FMO) activity, that in combination with a cytochrome P450 reductase, converts resokaempferol to fisetin, comprises an amino acid sequence having at least 70% sequence identity to the amino acid sequence of *Arabidopsis thaliana* FMO (gi 15241483; SEQ ID NO: 36). Alternatively, the FMO polypeptide may be selected from among *Arabidopsis thaliana* FMO (gi 15241483; SEQ ID NO: 36); *Petunia hybrida* FMO (gi 27151498; SEQ ID NO: 38); two subspecies of Fragaria x ananassa FMO 1 and 2 (gi 377550336; SEQ ID No: 40 and gi 332348707; SEQ ID No: 42); and *Malus x domestica* FMO (gi 237687728; SEQ ID No. 44); *Eutrema salsugineum* FMO (gi 567171580; SEQ ID NO: 46); *Capsella rub*ella FMO (gi 565459092; SEQ ID NO: 48); *Nicotiana tabacum* FMO (gi 583844041; SEQ ID NO: 50); and *Vitis vinifera* FMO (gi 526118008; SEQ ID NO: 52). In a further embodiment, the N-terminal sequence of any one of the above FMO polypeptides may be modified to enhance its expression, in particular expression in bacteria, by expressing an FMO polypeptide wherein the first four amino acids at the N-terminal of the respective polypeptide are deleted, and the subsequence two amino acids are substituted by the amino acid residues methionine followed by alanine.

The polypeptide having FMO activity may have at least 75, 80, 85, 90, 92, 95, 98 or 100% sequence identity to any one of SEQ ID NOs: 36, 38, 40, 42, 44, 46, 48, 50 and 52; more preferably at least 90% amino acid sequence identity to any one of SEQ ID NOs: 36, 46 and 48 encoding FMOat, FMOet and FMOcr respectively; or a polypeptide selected from any one of SEQ ID No: 36, 38 and 42 for FMOat, FMOph, and FMOfxa2 respectively.

A polypeptide having cytochrome P450 reductase (CPR) activity comprises an amino acid sequence having at least 70% sequence identity to the amino acid sequence of *Catharanthus roseus* CPR (gi 730125; SEQ ID NO: 54). Alternatively, the CPR polypeptide may be selected from among *Catharanthus roseus* CPR (gi 730125; SEQ ID NO: 54); *Arabidopsis thaliana* CPR (1) (gi 15233853; SEQ ID NO: 56) or CPR (2) (gi 332660338; SEQ ID NO: 58); *Petunia x hybrid* CPR (1) (gi 1726956; SEQ ID NO: 60) and CPR (2) (gi 71726954 ; SEQ ID NO: 62); *Vitis vinifera* CPR (gi 359485011; SEQ ID NO: 64); and *Capsella rubella* CPR (gi565451118; SEQ ID NO: 66).

The polypeptide having CPR activity may have at least 75, 80, 85, 90, 95, 98 or 100% sequence identity to any one of SEQ ID NOs: 54, 56, 58, 60, 62, 64 and 66; more preferably at least 70% amino acid sequence identity to any one of SEQ ID NOs: 54, 58, 60, and 64 for CPRcr, CPRat, CPRph, and CPRvv, respectively. In a further embodiment, a portion of the N-terminal sequence of the expressed CPR polypeptide may be deleted; for example from 60 to 80 amino acids (and any one integer thereof) from the N-terminus sequence may be deleted. More specifically, the first 71 amino acid residues may be deleted from *Catharanthus roseus* CPR (gi 730125; SEQ ID NO: 54); the first 74 amino acid residues may be deleted from *Arabidopsis thaliana* CPR (2) (gi 332660338; SEQ ID NO: 58); the first 73 amino acid residues may be deleted from Petunia x hybrid CPR (1) (gi 1726956; SEQ ID NO: 60); and the first 66 amino acid residues may be deleted from *Vitis vinifera* CPR (gi 359485011; SEQ ID NO: 64. The N-terminally deleted CPR polypeptide can be fused to the C-terminus of an FMO polypeptide for expression as a fusion protein.

The polypeptide having flavonoid 3'-monooxygenase (FMO) activity and the polypeptide having cytochrome P450 reductase activity, for conversion of resokaempferol to fisetin, may be expressed as a fusion protein, wherein the fusion protein has at least 75, 80, 85, 90, 92, 95, 98 or 100% sequence identity to any one of SEQ ID NOs: 156, 158, 160, 162, 164, 166; preferably 156 or 164.

II A micro-organism for production of fisetin from L- tyrosine and/or L-phenylalanine or fisetin pathway intermediates

The invention further provides a genetically modified micro-organism comprising transgenes whose expression provides a novel metabolic pathway for the synthesis of fisetin using, for example, either L- tyrosine and/or L-phenylalanine as a precursor. Where the precursor is L- tyrosine, this is converted into para (p)-coumaric acid and subsequently p-coumaroyl-coenzyme A (CoA) by tyrosine ammonia-lyase (TAL) and 4-Coumaroyl-CoA ligase (4CL), respectively (Figure 1). From here the biosynthetic pathway is directed to liquiritigenin by chalcone synthase (CHS) and chalcone reductase (CHR) that converts one molecule of p-

coumaroyl-CoA and three molecules of malonyl-CoA into isoliquiritigenin. Thus, produced isoliquiritigenin is isomerized into liquiritigenin in the presence of chalcone isomerase (CHI) (figure 1). The subsequence steps of the pathway for converting liquiritigenin to fisetin are described above in section I.

Where the precursor is L- phenylalanine, this is converted into cinnamic acid by phenylalanine ammonia-lyase (PAL), which is further converted into para (p)-coumaric acid and subsequently p-coumaroyl-coenzyme A (CoA) by cinnamate 4-hydroxylase (C4H) in conjunction with NADPH:cytochrome P450 reductase (CPR) followed by 4-Coumaroyl-CoA ligase (4CL), respectively (Figure 1). From here the biosynthetic pathway is directed to liquiritigenin by chalcone synthase (CHS) and chalcone reductase (CHR) that converts one molecule of p-coumaroyl-CoA and three molecules of malonyl-CoA into isoliquiritigenin. Thereby produced isoliquiritigenin is then isomerized into liquiritigenin in presence of chalcone isomerase (CHI) (figure 1). The subsequence steps of the pathway converting liquiritigenin to fisetin are described above in section I.

According to one embodiment, the invention provides a micro-organism capable of producing fisetin from an intermediate of the fisetin pathway selected from isoliquiritigenin, p-coumaryl-CoA, p-coumaric acid and L-tyrosine, that is characterized by having one or more genes encoding four enzymatic activities, F3H (EC 1.14.11.9); FLS (EC 1.14.11.23); FMO (EC 1.14.13.21), and CPR (EC 1.6.2.4), and as well as one or more genes encoding one or more of the additional five enzymatic activities, CHI (EC 5.5.1.6); CHS (EC 2.3.1.74), CHR (EC 2.3.1.170); 4CL (EC 6.2.1.12); and TAL (EC 4.3.1.23).

Thus a micro-organism of the invention, that is capable of producing fisetin using L-tyrosine as a precursor, is characterized by having one or more genes encoding the following nine enzymatic activities (TAL (EC 4.3.1.23); 4CL (EC 6.2.1.12); CHS (EC 2.3.1.74), CHR (EC 2.3.1.170); CHI (EC 5.5.1.6); F3H (EC 1.14.11.9); FLS (EC 1.14.11.23); FMO (EC 1.14.13.21), and CPR (EC 1.6.2.4).

Further, a micro-organism of the invention that is capable of producing fisetin using p-coumaric acid as a precursor is characterized by having one or more genes encoding the following eight enzymatic activities 4CL (EC 6.2.1.12); CHS (EC 2.3.1.74), CHR (EC 2.3.1.170); CHI (EC 5.5.1.6); F3H (EC 1.14.11.9); FLS (EC 1.14.11.23); FMO (EC 1.14.13.21), and CPR (EC 1.6.2.4).

Further, a micro-organism of the invention capable of producing fisetin using p-coumaryl-CoA as a precursor is characterized by having one or more genes encoding the following

seven enzymatic activities CHS (EC 2.3.1.74), CHR (EC 2.3.1.170); CHI (EC 5.5.1.6); F3H (EC 1.14.11.9); FLS (EC 1.14.11.23); FMO (EC 1.14.13.21), and CPR (EC 1.6.2.4).

Further, a micro-organism of the invention capable of producing fisetin using isoliquiritigenin as a precursor is characterized by having one or more genes encoding the following six enzymatic activities CHI (EC 5.5.1.6); F3H (EC 1.14.11.9); FLS (EC 1.14.11.23); FMO (EC 1.14.13.21), and CPR (EC 1.6.2.4).

According to an alternative embodiment, the invention provides a micro-organism capable of producing fisetin using cinnamic acid as precursor is characterized by having one or more genes encoding the following ten enzymatic activities CPR (EC 1.6.2.4); C4H (EC 1.14.13.11); 4CL (EC 6.2.1.12); CHS (EC 2.3.1.74), CHR (EC 2.3.1.170); CHI (EC 5.5.1.6); F3H (EC 1.14.11.9); FLS (EC 1.14.11.23); FMO (EC 1.14.13.21), and CPR (EC 1.6.2.4).

Further, a micro-organism capable of producing fisetin using L-phenylalanine as precursor is characterized by having one or more genes encoding the following enzymatic activities eleven PAL (EC 4.3.1.24); CPR (EC 1.6.2.4), C4H (EC 1.14.13.11); 4CL (EC 6.2.1.12); CHS (EC 2.3.1.74), CHR (EC 2.3.1.170); CHI (EC 5.5.1.6); F3H (EC 1.14.11.9); FLS (EC 1.14.11.23); FMO (EC 1.14.13.21), and CPR (EC 1.6.2.4).

One or more of the additional enzymatic activities and their corresponding enzymatic structural domains may be present in individual proteins, each encoded by a gene, or one or more of the enzymatic activities may be present in fusion proteins, where the fusion protein comprises more one or more active enzymatic structural domain encoded by a gene. The genes in the micro-organism of the invention that express a polypeptide having one or more active enzymatic domain, may be transgenes that are adapted for expression in the selected host cell, by employing a codon usage optimized for the given host cell, such codon optimization being well-known in the art. Nucleic acid molecules encoding a polypeptide having one or more enzymatic domain can be synthesized chemically, where the nucleic acid sequence of the molecule is selected to provide the codon usage optimized for the given host cell. The nucleic acid sequence of DNA molecules encoding each of the respective enzymatic activities of the fisetin pathway are exemplified in the sequence listing.

It should be appreciated that a micro-organism according to the described embodiments of the invention may express an endogenous copy of one or more of the genes encoding one or more polypeptide having an enzymatic activity associated with the invention; as well as a recombinant copy. In some embodiments, if a micro-organism has an endogenous copy of one or more of the genes, then a recombinant copy of these gene(s) will not be required. In some embodiments the micro-organism may endogenously express one or more enzymes of

the fisetin pathway described herein and may recombinantly express one or more other enzymes from the pathways for efficient production of fisetin.

In some embodiments, one or more of the genes encoding one or more polypeptide having an enzymatic activity associated with the invention is expressed in a recombinant expression vector. As used herein, a "vector" may be any of a number of nucleic acids into which a desired sequence or sequences may be inserted by restriction and ligation for transport between different genetic environments or for expression in a host cell. Vectors are typically composed of DNA, although RNA vectors are also available. Vectors include, but are not limited to: plasmids, fosmids, phagemids, virus genomes and artificial chromosomes. A suitable vector includes one which is able to replicate autonomously (self-replicating vector) or is integrated (integration vector) in the genome in a host cell, and which is further characterized by one or more endonuclease restriction sites at which the vector may be cut in a determinable fashion and into which a desired DNA sequence may be ligated such that the new recombinant vector retains its ability to replicate in the host cell. In the case of plasmids, replication of the desired sequence may occur many times as the plasmid increases in copy number within the host cell such as a host bacterium; or may occur just a single time per host before the host reproduces by mitosis. In the case of phage, replication may occur actively during a lytic phase or passively during a lysogenic phase.

When the one or more of the genes encoding one or more a polypeptide having an enzymatic activity associated with fisetin synthesis are expressed in a micro-organism of the invention, a variety of transcription control sequences (e.g., promoter/enhancer sequences) may be operably joined to the coding sequence encoding the respective polypeptide, such as to direct its expression. The promoter can be a native promoter, i.e., the promoter of the gene in its endogenous context, which provides normal regulation of expression of the gene. In some embodiments the promoter can be constitutive, i.e., the promoter is unregulated allowing for continual transcription of its associated gene. A variety of conditional promoters also can be used, such as promoters controlled by the presence or absence of a molecule.

The precise nature of the regulatory sequences needed for gene expression may vary between species or cell types, but shall in general include, as necessary, 5' non-transcribed and 5' non-translated sequences involved with the initiation of transcription and translation respectively, such as a TATA box, capping sequence, CAAT sequence, and the like. In particular, such 5' non-transcribed regulatory sequences will include a promoter region which includes a promoter sequence for transcriptional control of the operably joined gene.

Regulatory sequences may also include enhancer sequences or upstream activator sequences as desired. The vectors of the invention may optionally include 5' leader or signal sequences. The choice and design of an appropriate vector is within the ability and discretion of one of ordinary skill in the art.

Methods for introducing one or more transgene encoding the encoding polypeptides having one or more enzymatic activities into a host micro-organism of the invention is described in section V.

The synthesis of fisetin in cells of the micro-organism of the invention expressing the enzymatic activities (TAL, 4CL, CHS, CHR, F3H, FLS, FMO and CPR) and a substrate or co-substrate selected from L-tyrosine, liquiritigenin, garbanzol and resokaempferol is demonstrated in both bacterial cells (Example 17), and in fungal cells (Example 11). Additionally, in Example 6, the TAL, 4CL, CHS and CHR steps of the fisetin pathway are demonstrated in one host bacterial cell, and in Example 9 the F3H, FLS, FMO and CPR steps are demonstrated in a second host bacterial cell.

IIi Transgenic expression of a tyrosine ammonia-lyase

A polypeptide having tyrosine ammonia-lyase (TAL) activity, that converts L-tyrosine to para (p)-coumaric acid may be selected from among polypeptides having at least 70, 75, 80, 85, 90, 92, 95, 98 or 100% sequence identity to any one of *Rhodobacter sphaeroides* TALrs (gi 126464011; SEQ ID NO: 68); *Flavobacterium johnsoniae* TALfj (gi 146298870; SEQ ID NO: 70); *Saccharothrix espanaensis* TALse (gi 433607630; SEQ ID NO: 72); *Herpetosiphon aurantiacus* TALha-1 (gi 159898407; SEQ ID NO: 74) and (gi 159898927; SEQ ID NO: 76); *Rhodotorula mucilaginosa / Rhodotorula rubra* TALrm (gi 129592 SEQ ID NO: 78) and *Streptomyces sp. Tu 4128* TALBagA (gi 359308109; SEQ ID NO: 80).

The polypeptide having TAL activity may be selected from any one of SEQ ID NOs: 70, 74 and 76 for TALfj, TALha and TALha.

IIii Transgenic expression of phenyl ammonia lyase

A polypeptide having phenyl ammonia lyase (PAL) activity that converts L-phenylalanine into cinnamic acid comprises an amino acid sequence having at least 70, 75, 80, 85, 90, 95, 98 or 100% sequence identity to the amino acid sequence of *Arabidopsis thaliana* PAL (SEQ ID No: 150).

IIiii Transgenic expression of cinnamate 4-hydroxylase and NADPH:cytochrome P450 reductase

The hydroxylation of cinnamic acid to para-coumaric can be brought about by the overexpression of a homologous, or expression of a heterologous, cytochrome P450 reductase (CPR), in combination with cinnamate 4-hydroxylase.

A polypeptide having cinnamate 4-hydroxylase (C4H) activity, that (in conjunction with NADPH:cytochrome P450 reductase (CPR)) converts cinnamic acid into para (p)-coumaric acid has at least 70, 75, 80, 85, 90, 92, 95, 98 or 100% sequence identity to the amino acid sequence of *Arabidopsis thaliana* C4Hat (SEQ ID No 152).

The respective polypeptide having NADPH:cytochrome P450 reductase (CPR) activity, has at least 70, 75, 80, 85, 90, 92, 95, 98 or 100% sequence identity to the amino acid sequence of *Arabidopsis thaliana* ATR2 (SEQ ID No 58) or *Saccharomyces cerevisiae* cytochrome B5 (CYB5) (SEQ ID No. 154); or any one of the CPR polypeptides described above in section Iiii.

IIiv Transgenic expression of a Coumaroyl-CoA ligase

A polypeptide having Coumaroyl-CoA ligase (4CL) activity that converts para (p)-coumaric acid to p-coumaroyl-coenzyme A (CoA) comprises an amino acid sequence having at least 70% sequence identity to the amino acid sequence of *Petroselinum crispum* 4CLpc (gi 112801; SEQ ID NO: 82). Alternatively, the 4CL polypeptide may be selected from among *Petroselinum crispum* 4CLpc (gi 112801; SEQ ID NO: 82), *Lithospermum erythrorhizon* 4CLle (gi 1117778; SEQ ID NO: 84); *Streptomyces coelicolor* 4CLsc (gi 499339912; SEQ ID NO: 86); *Paulownia fortunei* 4CLpf (gi 219671340; SEQ ID NO: 88); *Fraxinus mandshurica* 4CLfm (gi 592923305; SEQ ID NO: 90); *Nicotiana tabacum* 4CLnt (gi 12229631; SEQ ID NO: 92); *Streptomyces violaceoruber* 4CLsv (gi 664339044; SEQ ID NO: 94); *Streptomyces coelicoflavus* ZG0656 4CLsc (gi 371543933; SEQ ID NO: 96); and *Streptomyces violaceorubidus* 4CLsv (gi 663145766; SEQ ID NO: 98).

The polypeptide having 4CL activity may have at least 75, 80, 85, 90, 92, 95, 98 or 100% sequence identity to any one of SEQ ID NOs: 82, 84, 86, 88, 90, 92, 94, 96, and 98; more preferably at least 75% amino acid sequence identity to any one of SEQ ID NOs: 82, 84, 88, 90 and 92 for 4CLpc, 4CLle, 4CLpf, 4CLfm, 4CLnt respectively.

IIv Transgenic expression of a chalcone synthase and chalcone reductase

One of more polypeptide having chalcone synthase (CHS) activity and/or chalcone reductase acitivity (CHR) whose activity in combination converts one molecule of p-coumaroyl-CoA and three molecules of malonyl-CoA into isoliquiritigenin. The polypeptide having CHS activity comprises an amino acid sequence having at least 85% sequence

identity to the amino acid sequence of *Petunia x hybrid* CHSph (gi7331152; SEQ ID No: 100). Alternatively, the CHS polypeptide may be selected from among of *Petunia x hybrid* CHSph (gi7331152; SEQ ID No: 100) *Arabidopsis thaliana* CHSat (gi 15240753; SEQ ID No: 102); *Fragaria x ananassa* CHSfxa (gi 71979908; SEQ ID No: 104); *Malus domestica* CHSmd (gi 213950516; SEQ ID No: 106); *Vitis vinifer*a CHSvv (gi 347664485; SEQ ID No: 108); *Hypericum androsaemum* CHSha (gi 62899809; SEQ ID No: 110); and *Nicotiana alata* CHSna (gi 239735962; SEQ ID No: 112).

The polypeptide having CHS activity may have at least 75, 80, 85, 90, 92, 95, 98 or 100% sequence identity to any one of SEQ ID NOs: 100, 102, 104, 106, 108, 110, and 112; more preferably at least 90% amino acid sequence identity to any one of SEQ ID NOs: 100, 104, 106, 108, 110, and 112 for CHSph, CHSfxa, CHSmd, CHSvv, CHSha,and CHSna respectively.

The polypeptide having CHR activity comprises an amino acid sequence having at least 70% sequence identity to the amino acid sequence of *Medicago sativa* CHRms (gi 563540; SEQ ID No: 114). Alternatively, the CHR polypeptide may be selected from among *Medicago sativa* CHRms (gi 563540; SEQ ID No: 114); *Arabidopsis thaliana* CHRat (gi 5080825; SEQ ID No: 116); *Fragaria x ananassa* CHRfxa (SEQ ID No: 118) and (gi 53988164; SEQ ID No: 120); *Malus domestica* CHRmd-1 (gi 658309920; SEQ ID No: 122) and CHRmd-2 (gi 658009769; SEQ ID No: 124); *Vitis vinifera* CHRvv (gi 297737567; SEQ ID No: 126); *Astragalus mongholicus* CHRam (gi 302129635; SEQ ID No: 128); ; and *Sesbania rostrata* CHRsr (gi 2792155; SEQ ID No: 130).

The polypeptide having CHR activity may have at least 75, 80, 85, 90, 95, 98 or 100% sequence identity to any one of SEQ ID NOs: 114, 118, 120, 122, 124, 126, 128 and 130; more preferably at least 85% amino acid sequence identity to any one of SEQ ID NOs: 114 and 128 for CHRms and CHRam respectively.

A fusion polypeptide having a domain having chalcone synthase (CHS) activity fused to a domain having chalcone reductase acitivity (CHR), and whose activity in combination converts one molecule of p-coumaroyl-CoA and three molecules of malonyl-CoA into isoliquiritigenin is demonstrated in Example 15 and 16. Surprisingly the expression of the enzymes CHS and CHR in *E. coli* as a fusion protein, rather than separately, results in a significant increase in *in vivo* flux towards liquiritigenin.

IIvi Transgenic expression of a chalcone isomerase

A polypeptide having chalcone isomerase (CHI) activity that isomerises isoliquiritigenin into liquiritigenin comprises an amino acid sequence having at least 70% sequence identity to the amino acid sequence of *Medicago sativa* CHIms (gi 116134; SEQ ID No: 132). Alternatively, the CHI polypeptide may be selected from among *Medicago sativa* CHIms (gi 116134; SEQ ID No: 132) and (gi 166398; SEQ ID No: 134); *Lotus japonicus* CHIlj (gi 28804193; SEQ ID No: 136); *Medicago truncatula* CHImt (gi 357444967; SEQ ID No: 138); *Glycine max* CHIgm (gi 114199183; SEQ ID No: 140); *Glycyrrhiza uralensis* CHIgu (gi 122725493; SEQ ID No: 142); *Pueraria montana* var. *lobata* CHIpm (gi 5921724; SEQ ID No: 144); *Phaseolus vulgaris* CHIpv (gi 593592189; SEQ ID No: 146); and *Astragalus mongholicus* CHIam (gi 334851451; SEQ ID No: 148).

The polypeptide having CHI activity may have at least 75, 80, 85, 90, 95, 98 or 100% sequence identity to any one of SEQ ID NOs: 132, 134, 136, 138, 140, 142, 144, 146 and 148; more preferably at least 80% amino acid sequence identity to any one of SEQ ID NOs: 132, 134, 136, 138, 140, 142, and 146 for CHIms, CHImt, CHIgm, CHIgu, and CHIpv respectively.

III A micro-organism for production of garbanzol and/or resokaempferol from L-tyrosine and/or L-phenylalanine

The invention further provides a genetically modified micro-organism comprising genes whose expression provides a novel metabolic pathway for the synthesis of garbanozol and/or resokaempferol from L- tyrosine and/or L-phenylalanine.

Where the precursor is L- tyrosine, this is converted into the intermediate para (p)-coumaric acid by tyrosine ammonia-lyase (TAL; EC 4.3.1.23). Alternatively, where the precursor is L-phenylalanine, this is converted into cinnamic acid by phenylalanine ammonia-lyase (PAL; EC 4.3.1.24), which is further converted into para (p)-coumaric acid by cinnamate 4-hydroxylase (C4H; EC 1.14.13.11) in conjunction with NADPH:cytochrome P450 reductase (Figure 1).

The subsequent steps of the biosynthetic pathway are common for the two precursors (L-tyrosine and/or L-phenylalanine), whereby the intermediate para (p)-coumaric acid is converted to p-coumaroyl-coenzyme A (CoA) by 4-Coumaroyl-CoA ligase 4CL (EC 6.2.1.12); and then one molecule of p-coumaroyl-CoA and three molecules of malonyl-CoA is converted into isoliquiritigenin by chalcone synthase (CHS; EC 2.3.1.74) and chalcone reductase (CHR; EC 2.3.1.170). Thus produced isoliquiritigenin is isomerized into liquiritigenin in presence of chalcone isomerase (CHI; EC 5.5.1.6), and liquiritigenin is then

converted into garbanzol by the enzyme flavanone 3-hydroxylase (F3H EC 1.14.11.9) (figure 1). One additional step in the pathway catalysed by flavonol synthase (FLS; EC 1.14.11.23) converts garbanzol into resokaempferol.

One or more of each of the respective enzymatic activities and their corresponding enzymatic domains, may be present in individual proteins, each encoded by a gene, or one or more of the enzymatic activities may be present in fusion proteins, where the fusion protein comprises more than one active enzymatic domain encoded by a gene. The genes in the micro-organism of the invention that express a polypeptide comprising one or more active enzymatic domain, may be transgenes that are adapted for expression in the selected host cell, by employing a codon usage optimized for the given host cell, such codon optimization being well-known in the art. Nucleic acid molecules encoding a polypeptide having one or more enzymatic activities can be synthesized chemically, where the nucleic acid sequence of the molecule is selected to provide the codon usage optimized for the given host cell. The nucleic acid sequence of DNA molecules encoding each of the respective enzymatic activities of the fisetin pathway are exemplified in the sequence listing.

Methods for introducing one or more transgene encoding the encoding polypeptides having one or more four enzymatic activities into a host micro-organism of the invention is described in section V.

The synthesis of garbanozol or resokaempferol in cells of the micro-organism of the invention is demonstrated in (Example 7; figure 2).

IIIi Transgenic expression of enzymes for the production of garbanozol and/or resokaempferol

Suitable enzymes for the production of garbanozol and/or resokaempferol are:

Tyrosine ammonia-lyase, TAL (EC 4.3.1.23), to convert L-tyrosine to para (p)-coumaric acid, as described in section IIi: OR

phenylalanine ammonia-lyase, PAL (EC 4.3.1.24), to convert L-phenylalanine to cinnamic acid, as described in section IIii, AND

cinnamate 4-hydroxylase, C4H (EC 1.14.13.11) and NADPH:cytochrome P450 reductase, CPR (EC 1.6.2.4), to convert cinnamic acid into para (p)-coumaric acid, as described in section IIiii.

The subsequent steps in the production of garbanozol and/or resokaempferol are performed with the following enzymes:

4-Coumaroyl-CoA ligase, 4CL (EC 6.2.1.12) to convert para (p)-coumaric acid to p-coumaroyl-coenzyme A (CoA) as described in section IIiv, AND

chalcone synthase, CHS (EC 2.3.1.74), and chalcone reductase, CHR (EC 2.3.1.170), for conversion of one molecule of p-coumaroyl-CoA and three molecules of malonyl-CoA into isoliquiritigenin, as described in section IIv; AND

chalcone isomerase, CHI (EC 5.5.1.6), to isolermise isoliquiritigenin into liquiritigenin, as described in section IIvi; AND

flavanone 3-hydroxylase, F3H (EC 1.14.11.9) to convert liquiritigenin into garbanzol, as described in section Ii.

The additional enzyme required for the production of resokaempferol is:

flavonol synthase, FLS (EC 1.14.11.23), to convert garbanzol into resokaempferol, as described in section Iii.

IV Micro-organisms comprising a pathway for garbanozol and/or resokaempferol and/or fisetin synthesis

The micro-organism according to the invention, comprising a pathway for synthesis of garbanozol and/or resokaempferol and/or fisetin, may be a fungus, and more specifically a filamentous fungus belonging to the genus of *Aspergillus*, e.g. *A. niger, A. awamori, A. oryzae, A. nidulans*; a yeast belonging to the genus of Saccharomyces, e.g. *S. cerevisiae, S. kluyveri, S. bayanus, S. exiguus, S. sevazzi, S. uvarum*; a yeast belonging to the genus *Kluyveromyces*, e.g. *K. lactis K. marxianus var. marxianus, K. thermotolerans*; a yeast belonging to the genus Candida, e.g. *C. utilis C. tropicalis, C. albicans, C. lipolytica, C. versatilis*; a yeast belonging to the genus Pichia, e.g. *P. stipidis, P. pastoris, P. sorbitophila*, or other yeast genera, e.g. *Cryptococcus, Debaromyces, Hansenula, Pichia, Yarrowia, Zygosaccharomyces* or *Schizosaccharomyces*. Concerning other micro-organisms a non-exhaustive list of suitable filamentous fungi is supplied: a species belonging to the genus *Penicillium, Rhizopus, Fusarium, Fusidium, Gibberella, Mucor, Mortierella,* and *Trichoderma*.

Alternatively, the micro-organism comprising a pathway for garbanozol and/or resokaempferol and/or fisetin synthesis according to the invention may be a bacterium, a non-exhaustive list of suitable bacteria is given as follows: a species belonging to the genus *Bacillus*, a species belonging to the genus *Escherichia*, a species belonging to the genus *Lactobacillus*, a species belonging to the genus *Lactococcus*, a species belonging to the

genus *Corynebacterium*, a species belonging to the genus *Acetobacter*, a species belonging to the genus *Acinetobacter*, a species belonging to the genus *Pseudomonas*, etc.

The preferred micro-organisms of the invention may be *S. cerevisiae, E. coli, L. lactis* or *L. plantarum.*

V Methods for producing a micro-organism of the invention

Integration and self-replicating vectors suitable for cloning and introducing one or more gene encoding one or more a polypeptide having an enzymatic activity associated with fisetin synthesis in a micro-organism of the invention are commercially available and known to those skilled in the art (see, e.g., Sambrook et al., Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press, 1989). Cells of a micro-organism are genetically engineered by the introduction into the cells of heterologous DNA (RNA). Heterologous expression of genes encoding one or more polypeptide having an enzymatic activity associated with fisetin synthesis in a micro-organism of the invention is demonstrated in the Examples.

A nucleic acid molecule, that encodes one or more a polypeptide having an enzymatic activity associated with garbanozol and/or resokaempferol and/or fisetin synthesis according to the invention, can be introduced into a cell or cells and optionally integrated into the host cell genome using methods and techniques that are standard in the art. For example, nucleic acid molecules can be introduced by standard protocols such as transformation including chemical transformation and electroporation, transduction, particle bombardment, etc. Expressing the nucleic acid molecule encoding the enzymes of the claimed invention also may be accomplished by integrating the nucleic acid molecule into the genome.

VI A method for producing garbanozol and/or resokaempferol and/or fisetin

Fisetin can be produced using a micro-organism of the invention by introducing the micro-organism into a culture medium; providing the culture with one or more suitable substrate and /or co-substrate for garbanozol and/or resokaempferol and/or fisetin biosynthesis; and finally recovering the garbanozol, resokaempferol, and/or fisetin produced by the culture, as illustrated in the Examples.

Many compounds throughout the entire fisetin pathway are suitable as substrate or co-substrate for production of fisetin, namely L-tyrosine, L-phenylalanine, *p*-coumaric acid, liquiritigenin, garbanzol and resokaempferol. Suitable substrates or co-substrates for the synthesis of garbanozol and/or resokaempferol includes L-tyrosine, L-phenylalanine, *p*-coumaric acid, liquiritigenin. Furthermore, micro-organisms of the invention for production

of garbanozol, resokaempferol, and/or fisetin can be engineered in order to use glucose, L-phenyl-alanine or cinnamic acid as substrate, including *E. coli, S.cerevisiae* and *Lactococcus lactis*.

Where garbanozol and/or resokaempferol, and/or fisetin is secreted by the micro-organism of the invention, these flavonoid products can be recovered from the growth medium; and where the flavonoid product is an intracellular product, it can be recovered from cells of the micro-organism of the invention by permeabilization of cell membranes combined with extraction of the flavonoid, employing standard methods for flavonoid extraction, including solvent extraction, and subsequent purification, as illustrated in the examples.

VII A method of detecting production of garbanozol, resokaempferol, or fisetin production

Methods for detecting and quantifying garbanozol, resokaempferol, or fisetin produced by a micro-organism of the invention include high performance liquid chromatography (HPLC) combined with UV detection to identify and quantify garbanozol, resokaempferol, or fisetin and its biosynthetic precursors, relative to a set of standards for each step of their biosynthetic pathway, as described herein, as illustrated in the examples.

EXAMPLES

Example 1. Construction of recombinant plasmids for functional expression of an enzymatic pathway for the conversion of L-tyrosine to liquiritigenin in *E. coli*

This part of the pathway was constructed using three different plasmids pDFDuet-1, pETDuet-1 and pRSFDuet-1(Novagen).

Plasmid pCDF4CL was constructed by cloning Pc4cL-2 cDNA between *EcoR*V and *Kpn*I in vector pCDFDuet-1 under the T7-promoter in the multiple cloning site 2 (MCS2).

Amplification of Pc4cL-2 cDNA encoding a 4-Coumaroyl-CoA ligase (4CL) enzyme from *Petroselinum crispum* (4CLpc; gi 112801; accession no. X13325), was carried out using primer pair pCDF-Pc4cL2-F and pCDF-Pc4cL2-R (table 1).

A synthetic gene encoding tyrosine ammonia lyase (TAL) from *Rhodobacter sphaeroides* (TALrs, gi 126464011; genBank accession no. YP_001045124), codon optimized for expression in *E. coli,* was amplified using primer pairs TAL_F and TAL_R (table 1) and subsequently cloned, using Gibson assembly, into the linearized vector pCDF4CL2 under the T7-promoter in the multiple cloning site 1 (MCS1), creating pCDFTALrs4CL2pc. The pCDF4CL2 vector was linearized (digested) with restriction nuclease enzymes *Nco*I and *Sal*I.

Synthetic genes encoding chalcone reductase (CHR) from *Astragalus mongholicus* (CHRam, gi 302129635; genBank accession no. HM357239) and *Medicago Sativa* (CHRms, gi 563540; genBank accession no. CAA57782) were codon optimized for expression in *E. coli* and subsequently cloned (utilizing Gibson assembly) into a pRSFDuet-1 vector under the T7 promoter in the MCS1 using primer pairs CHRam_F and CHRam_R for pRSFCHRam and CHRms_F and CHRms_R for pRSFCHRms, respectively (table 1).

Plasmid pETCHSphCHIms harboring genes encoding a chalcone synthase (CHS) from Petunia hybrid (CHSph, gi 7331152; genBank accession no. AF233638 and a chalcone isomerase (CHI) from *Medicago Sativa* (CHIms, gi: 166398; genBank accession no. M91079) was created by subcloning *chs* between EcoR V and Kpn I and *chi* between BamH I and Pst I sequentially in vector pETDuet-1 under the T7 promoter in the MCS1 and MCS2, respectively. Primer pairs used for amplification were CHS-F and CHS-R for CHS and CHI F and CHI R for CHI (table 1). Lastly plasmids were transformed into *E. coli* BL21(DE3) creating strain ST2am (pCDFTALrs4CL2pc and pETCHSphCHIms and pRSFCHRam) and ST2ms (pCDFTALrs4CL2pc and pETCHSphCHIms and pRSFCHRms).

Example 2. Construction of recombinant plasmids for functional expression of an enzymatic pathway for the conversion of resokaempferol to fisetin in *E. coli*

In order to construct the recombinant plasmid for functional expression of various FMO (P450) candidates in combination with its redox partner P450 reductase (CPR), genes encoding these two enzymes were fused together. Initially, 69 codons were removed from the 5' end of the *cpr* gene and replaced by 5' GTCGAC 3' (SalI restriction site). Thus, *cpr* from *Catharanthus roseus* encoding CPR (CPRcr, gi 730125; genBank accession no. X69791) was codon optimized for expression in *E. coli*, truncated and subsequently cloned into the pACYCDuet-1 vector in the MCS1 which was digested by restriction nuclease enzymes SalI and KpnI, creating pACYCcpr. CPRcr was amplified using primer pair CPR_CR_F and CPR_CR_R (table 1).

The first 4 codons of the 5' end (N-terminal) of various genes encoding FMO were replaced by ATG GCG, followed by the removal of the stop codon TAA in the 3' end (C-terminal) of the respective gene, which was replaced by GGGTCGAC, a linker region containing a SalI restriction site (underlined in sequences). The truncated genes encoding flavonoid 3'-monooxygenase (FMO) were then fused using nuclease enzymes NcoI and SalI, together with the *cpr* in pACYCcpr, respectively. As a reference, a gene encoding a flavonoid 3', 5'-hydroxylase (F3'5'H) from *C. roseus* described by Leonard et al., 2006 were fused to CPRcr and cloned into pACYCDuet-1 in a similar manner using Gibson assembly. The following genes encoding FMO and primer pairs were used for amplification:

FMO from *A. thaliana* (FMOat, gi 15241483; genBank accession no. AAF60189) was amplified using primer pair FMOat_F and FMOat_R; FMO from *Fragaria x ananassa* (FMOfxa1, gi 377550336; genBank accession no. BAL63027), was amplified using primer pair FMOfxa1_F and FMOfxa1_R; FMO from *Fragaria x ananassa* (FMOfxa2, gi 332348707; genBank accession no. AEE60886) was amplified using primer pair FMOfxa2_F and FMOfxa2_R; FMO from *Malus x domestica* (FMOmxd, gi 237687728; genBank accession no. ACR14867) was amplified using primer pair FMOmxa_F and FMOmxa_R FMO from *Petunia hybrida* (FMOph, gi 27151498; genBank accession no. AAD56282) was amplified using primer pair FMOph_F and FMOph_R and f3'5'h and CPR from *C. roseus* (F3'5'Hcr, gi 3954807; genBank accession no. CAA09850, was amplified using primer pair F3'5'Hcr_F and FMO5'Hcr_R and CPR_CR_GIB_F and CPR_CR_GIB_R, respectively (table 1). Genes encoding fusion proteins having FMO and CPR activity were amplified from the following: Arabidopsis thaliana FMO (gi 15241483) and *Catharanthus roseus* CPR (encoding gi 730125) (SEQ ID No. 156); *Fragaria ananassa* FMOfxa1 (encoding gi 377550336) and *Catharanthus roseus* CPR (encoding gi 730125) (SEQ ID NO 158); Fragaria *ananassa* FMOfxa2 (encoding gi 332348707) and *Catharanthus roseus* CPR (encoding gi 730125) (SEQ ID NO 160); *Malus domestica* FMOmxd (gi 237687728) (SEQ ID No. 162); *Petunia hybrid* FMO (encoding gi 27151498) and *Catharanthus roseus* CPR (encoding gi 730125) (SEQ ID No 164); *Catharanthus roseus* FMO (F3'5'H) (gi 3954807) and *Catharanthus roseus* CPR (encoding gi 730125) (SEQ ID No 166). *E. coli* DH5α (Invitrogen) was used for plasmid cloning and propagation.

Lastly plasmids were transformed into *E. coli* BL21(DE3) creating strain ST4at (pACYCFMOat); ST4fxa1 (pACYCFMOfxa1); ST4fxa2 (pACYCFMOfxa2); ST4mxd (pACYCFMOmxd); ST4ph (pACYCFMOph) and ST4cr (pACYCF3'5'Hcr), which are listed in Table 4.

Example 3. Construction of recombinant plasmids for functional expression of an enzymatic pathway for the conversion of liquiritigenin to fisetin in *E. coli*

The pathway from liquiritigenin to fisetin was expressed on two plasmids pCDFDuet-1 and pACYCDuet (Novagen).

The gene encoding flavanone 3-hydroxylase (F3H) from *Arabidopsis thaliana* (F3Hat, gi 15230433; genBank accession no. NM_114983.3) was amplified using primer pairs AT3G51240 F and AT3G51240 R (table 1). pCDFDuet and the PCR amplicon were digested with *Nco*I and *EcoR*I for two hours at 37°C . The PCR amplicon was subsequently cloned into the linearized vector pCDFDuet using T4 ligase (Fermentas) under the T7-promoter in the multiple cloning site 1 (MCS1), creating pCDFF3Hat.

The gene encoding flavonol synthase (FLS) from *Arabidopsis thaliana* (FLSat, gi 334187530; genBank accession no. NM_001203337.1) was amplified using primer pairs FLS1 F and FLS1 R (table 1). pCDFF3Hat and the PCR amplicon were digested with *Nde*I and *Kpn*I for two hours at 37°C . The PCR amplicon was subsequently cloned into the linearized vector pCDFF3Hat using T4 ligase (Fermentas) under the T7-promoter in the multiple cloning site 2 (MCS2), creating pCDFF3HatFLSat. *E. coli* DH5α (Invitrogen) was used for plasmid cloning and propagation.

Construction of plasmids for expression of P450's fused with CPR (pACYCFMOat; pACYCFMOfxa; pACYCFMOfxa2; pACYCFMOmxd; pACYCFMOph and pACYCF3'5'Hcr) is described in example 2. Lastly plasmids were transformed into *E. coli* BL21(DE3) creating strain ST5at (pCDFF3HatFLSat and pACYCFMOat); ST5fxa1 (pCDFF3HatFLSat and pACYCFMOfxa1); ST5fxa2 (pCDFF3HatFLSat and pACYCFMOfxa2); ST5mxd (pCDFF3HatFLSat and pACYCFMOmxd); ST5ph (pCDFF3HatFLSat and pACYCFMOph) and ST5cr (pCDFF3HatFLSat and pACYCF3'5'Hcr). The resultant strains are listed in Table 5.

Table 1 Primers for *E. coli* pathway construction

Primers	Oligonucleotide sequences (5'-3')	Restriction site	SEQ ID No.
TAL_R	TAAGCATTATGCGGCCGCAAGCTTGTTAAACCGGACTCTGTTGCA	NA	167
TAL_F	TAGAAATAATTTTGTTTAACTTTAATAAGGAGATATACCATGCTGGCAATGAGCCCTCC	NA	168
pCDF-Pc4cL2-F	GGGGGGGATATCGGATGGGAGACTGTGTAGCACCCAAAG	*EcoR*V	169
pCDF-Pc4cL2-R	CCCCCCGGTACCCCTTATTTGGGAAGATCACCGGATGCT	*kpn*I	170
CHS-F	GGGGGGATCCGGATGGTGACAGTCGAGGAGTATCGTA	*Eco*RI	171
CHS-R	CCCCCTGCAGCCTTAAGTAGCAACACTGTGGAGGACA	*Sal*I	172
CHI F	GGGGGGGATATCATGGCTGCATCAATCACCGCAATCA	*EcoR*V	173
CHI R	CCCCCCGGTACCTCAGTTTCCAATCTTGAAAGCACCC	*kpn*I	174
CHRms_F	TAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACCATGGGTAGCGT	NA	175
CHRms_R	TAAGCATTATGCGGCCGCAAGCTTGTTAGTCATCATACAGATCATTC	NA	176
CHRam_F	TAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACCATGGGTAGCGTTAGCGTTGA	NA	177
CHRam_R	TAAGCATTATGCGGCCGCAAGCTTGTTAGATTTCGTCATCCCACAG	NA	178
AT3G51240 F	CCATGGCAATGGCTCCAGGAACTTTGA	*Nco*I	179
AT3G51240 R	GAATTCCTAAGCGAAGATTTGGTCGACA	*Eco*RI	180
FLS1 F	CATATGGAGGTCGAAAGAGTCCAAGACATT	*Nde*I	181
FLS1 R	AGGTACCTCAATCCAGAGGAAGTTTATTGAG	*Kpn*I	182
CPR_CR_F	AGAGAGGTCGACTAGCAGCGGTAGTGGTAAAAAAGTTG	*Sal*I	183
CPR_CR_R	AGAGAGGGTACCTTACCAAACGTCACGCAGATAACG	*kpn*I	184
FMOat_F	GGGGCCATGGCGACCATTCTGCTGGC	*nco*I	185
FMOat_R	GGGG*GTCGAC*CCACCTGAACCCAGACCATAAA	*Sal*I	186
FMOfxa1_F	GGGGCCATGGCGATTACCCTGCTGGT	*nco*I	187
FMOfxa1_R	GGGGGTCGACCCGCTGGTTTTATATGCATGCG	*Sal*I	188

FMOfxa2_F	GGGGCCATGGCGATTACCCTGCTGGT	*nco*I	189
FMOfxa2_R	GGGGGTCGACCCTGAGCTGCTGGTTTTATATG	*Sal*I	190
FMOmxd_F	GGGGCCATGGCGTTCACCGTGGTGTT	*nco*I	191
FMOmxd_R	GGGGGTCGACCCGCTGCTGCTTGCATTATATG	*Sal*I	192
FMOph_F	GGGGCCATGGCGATTCTGTATACCGT	*nco*I	193
FMOph_R	GGGGGTCGACCCACCAATATATGCCTGGGCTT	*Sal*I	194
F3'5'Hcr_F	TAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACCATGGCGGTGCTGT TTGATTTTCTGGCAGC	NA	195
F3'5'Hcr_R	CAACTTTTTTACCACTACCGCTGCTAGTCGACCCCGGGCTATATGCATTAATC GGC	NA	196
CPR_CR_GIB_F	GCCGATTAATGCATATAGCCCGGGGTCGACTAGCAGCGGTAGTGGTAAAAA AGTTG	NA	197
CPR_CR_GIB_R	TAAGCATTATGCGGCCGCAAGCTTGTTACCAAACGTCACGCAGATAACG	NA	198

Example 4 Construction of recombinant plasmids for functional expression of a fisetin pathway in *Saccharomyces cerevisiae*

The following genes were cloned for the functional expression of a fisetin pathway in *Saccharomyces cerevisiae*: *Petroselinum crispum* 4-CL (encoding gi 112801; SEQ ID NO: 82), *Petunia hybrid* CHS (encoding gi 7331152; SEQ ID NO: 100), *Medicago sativa* CHR (encoding gi 563540; SEQ ID NO 126), *Medicago sativa* CHI (encoding gi 116134; SEQ ID NO 146), *Arabidopsis thaliana* F3H (encoding gi 15230433 SEQ ID NO 2), *Arabidopsis thaliana* flavonol synthase FLS (encoding gi 334187530; SEQ ID NO 18), fusion of *Petunia hybrid* FMO (encoding gi 27151498) and *Catharanthus roseus* CPR (encoding gi 730125) (SEQ ID No 164) or fusion of *Fragaria ananassa* FMOfxa2 (encoding gi 332348707) and *Catharanthus roseus* CPR (encoding gi 730125) (SEQ ID NO 162). To enable *de novo* biosynthesis of p-coumaric acid from tyrosine, we additionally cloned *Flavobacterium johnsoniae* TAL (encoding gi 146298870; SEQ ID NO 74), phenylalanine ammonia lyase (PAL2) from *Arabidopsis thaliana* codon-optimized for *S. cerevisiae* (gi 15231778; SEQ ID NO 150), cinnamate-4-hydroxylase (C4H) from *A. thaliana* codon-optimized for *S. cerevisiae* (gi 15224514;SEQ ID NO 152), cytochrome P450 reductase (ATR2) from *A. thaliana* codon-optimized for *S. cerevisiae* (encoding gi 15234668;SEQ ID NO 58), and cytochrome B5 (CYB5) from *S. cerevisiae* (encoding gi: 398364811;SEQ ID NO 154). To improve the biosynthesis of tyrosine and phenylalanine, we overexpressed a feedback-inhibition resistant allele of 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase $ARO4^{K229L}$ and a feedback-inhibition resistant allele of chorismate mutase $ARO7^{G141S}$ from *S. cerevisiae.*

The gene fragments carrying the genes and overhangs for USER-cloning were generated by PCR amplification. The PCR mix contained: 28 µl water, 10 µl high fidelity Phusion® polymerase buffer (5x), 5 µl 2mM dNTP, 1 µl Phusion® polymerase, 2.5 µl forward primer at 10 µM concentration, 2.5 µl reverse primer at 10 µM concentration, and 1 µl DNA template. The cycling program was: 95°C for 2 min, 30 cycles of [95°C for 10 sec, 50°C for 20 sec, 68°C for 2 min], 68°C for 5 min, pause at 10°C. The gene fragments were resolved on 1% agarose gel containing SYBR®-SAFE (Invitrogen) and purified using NucleoSpin® Gel and PCR Clean-up kit (Macherey-Nagel). The primer sequences are listed in Table 2. The gene and promoter fragments as well as primers and templates used for their PCR amplification are listed in Table 3. The terminators were already present on the expression plasmids.

Table 2 Primers for *Saccharomyces cerevisiae* pathway construction

Primer name	Primer sequence, 5'->3'	SEQ ID No.
Pc_4Cl_U2_fw	ATCTGTCAU AAAACA ATG GGA GAC TGT GTA GCA C	199
Pc_4Cl_U2_rv	CACGCGAU TCA TTA TTT GGG AAG ATC ACC GGA TG	200
Ph_CHS_U1_fw	AGTGCAGGU AAAACA ATG ACC ATG GTT ACC GTT GAA G	201
Ph_CHS_U1_rv	CGTGCGAU TCA TTA GGT TGC AAC GCT ATG CAG	202
Ms_CHI_U1_fw	AGTGCAGGU AAAACA ATG ACC ATG GCA GCA AGC	203
Ms_CHI_U1_rv	CGTGCGAU TCA TTA GTT GCC GAT TTT AAA GGC ACC	204
Ms_CHR_U2_fw	ATCTGTCAU AAAACA ATG ACC ATG GGT AGC GTT G	205
Ms_CHR_U2_rv	CACGCGAU TCA TTA GTC ATC ATA CAG ATC ATT CAG ACC	206
At_F3H_U2_fw	ATCTGTCAU AAAACA ATG GCT CCA GGA ACT TTG AC	207
At_F3H_U2_rv	CACGCGAU TCA CTA AGC GAA GAT TTG GTC GAC AG	208
At_FLS_U1_fw	AGTGCAGGU AAAACA ATG GAG GTC GAA AGA GTC C	209
At_FLS_U1_rv	CGTGCGAU TCA TCA ATC CAG AGG AAG TTT ATT GAG C	210
Ph_FMO_U1_fw	AGTGCAGGU AAAACA ATG GCG ATT CTG TAT ACC GTG	211
Fa_FMO_U1_fw	AGTGCAGGU AAAACA ATG GCG ATT ACC CTG CTG	212
Cr_CPR_U1_rv	CGTGCGAU TCA TTA CCA AAC GTC ACG CAG ATA AC	213
Fsp_Tal_U1_fw	AGTGCAGGU AAAACA ATG AAC ACC ATC AAC GAA TAT CTG AGC	214
Fsp_Tal_U1_rv	CGTGCGAU TTA ATT GTT AAT CAG GTG	215
Sc_Aro4_U2_fw	ATCTGTCAU AAAACA ATG AGT GAA TCT CCA ATG TTC G	216
Sc_Aro4_U2_rv	CACGCGAU TCA TTT CTT GTT AAC TTC TCT TCT TTG	217
Sc_Aro7_U1_fw	AGTGCAGGU AAAACA ATG GAT TTC ACA AAA CCA GAA AC	218
Sc_Aro7_U1_rv	CGTGCGAU TCA CTC TTC CAA CCT TCT TAG CAA G	219
PTEF1_fw	ACCTGCACU TTGTAATTAAAACTTAG	220
PTEF1_rv	CACGCGAU GCACACACCATAGCTTC	221
PPGK1_rv	ATGACAGAU TTGTTTTATATTTGTTG	222
AtPAL2_U1_fw	AGTGCAGGU AAAACAATGGATCAAATC	223
AtPAL2_U1_rv	CGTGCGAU TCAGCAGATAGGAATAGG	224
AtC4H_U2_fw	ATCTGTCAU AAAACAATGGACTTGTTGTTGTTG	225
AtC4H_U2_rv	CACGCGAU TCAACAGTTTCTTGGCTT	226

cyb5_U1_fw	AGTGCAGGU AAAACAATGCCTAAAGTTTACAGTTACC	227
cyb5_U1_rv	CGTGCGAU TCATTCGTTCAACAAATAATAAGC	228
AtATR2_U2_fw	ATCTGTCAU AAAACAATGTCCTCCTCTTCTTCATCATCCACC	229
AtATR2_U2_rv	CACGCGAU TCACCAGACATCTCTCAA	230

* Kozak sequence AAAACA is underlined.

Table 3. Gene and promoter fragments amplified by PCR

Fragment name	Gene	Fw Primer	Rv primer	Template DNA
Pc_4Cl->	4-Coumaroyl-CoA ligase from *Petroselinum crispum*	Pc_4Cl_U2_fw	Pc_4Cl_U2_rv	pCDFTALrs4CL2pc
Ph_CHS<-	Chalcone synthase from *Petunia hybrid*	Ph_CHS_U1_fw	Ph_CHS_U1_rv	pETCHSphCHIms
Ms_CHI<-	Chalcone isomerase from *Medicago sativa*	Ms_CHI_U1_fw	Ms_CHI_U1_rv	pETCHSphCHIms
Ms_CHR->	6'-deoxychalcone synthase from *Medicago sativa*	Ms_CHR_U2_fw	Ms_CHR_U2_rv	pRSFCHRms
At_F3H->	Flavanone 3-hydroxylase from *Arabidopsis thaliana*	At_F3H_U2_fw	At_F3H_U2_rv	pCDFF3HatFLSat
At_FLS<-	Flavonol synthase from *Arabidopsis thaliana*	At_FLS_U1_fw	At_FLS_U1_rv	pCDFF3HatFLSat
Ph_FMO-Cr_CPR<-	Fusion of flavonoid 3'-monooxygenase from *Petunia hybrid* and cytochrome P450 reductase from *Catharanthus roseus*	Ph_FMO_U1_fw	Cr_CPR_U1_rv	pACYCFMOph
Fa_FMO-Cr_CPR<-	Fusion of flavonoid 3'-monooxygenase from *Fragaria ananassa* and cytochrome P450 reductase from *Catharanthus roseus*	Fa_FMO_U1_fw	Cr_CPR_U1_rv	pACYCFMOat
TALfj <-	Tyrosine ammonia lyase from *Flavobacterium sp.*	Fsp_Tal_U1_fw	Fsp_Tal_U1_rv	Synthetic gene commercially acquired from gene art
Sc_Aro7_G141S<-	3-Deoxy-D-arabino-heptulosonate7-phosphate (DAHP) synthase from *S. cerevisiae*, point-mutated allele	Sc_Aro7_U1_fw	Sc_Aro7_U1_rv	pESC-Ura-ARO7_G141S
Sc_Aro4_K229L->	Chorismate mutase from *S. cerevisiae*,	Sc_Aro4_U2_fw	Sc_Aro4_U2_rv	pESC-His-ARO4_K229L

	point-mutated allele			
<-PTEF1	*TEF1* promoter from *S. cerevisiae*	PTEF1_fw	PTEF1_rv	pSP-GM1 (Partow *et al.*, 2010)
<-PTEF1-PPGK1->	Fused *TEF1* and *PGK1* promoters from *S. cerevisiae*	PTEF1_fw	PPGK1_rv	pSP-GM1 (Partow *et al.*, 2010)

The parent integrative vectors from the EasyClone (Jensen *et al.,* 2014) and/or EasyClone2.0 (Stovicek *et al.,* 2015) sets for *Saccharomyces cerevisiae* were linearized with FastDigest® AsiSI (Fermentas) for 1 hour at 37°C and nicked with Nb.BsmI for 1 hour at 37°C. The resulting linearized nicked DNA was purified from the solution using NucleoSpin® Gel and PCR Clean-up kit (Macherey-Nagel). The integrative expression vectors were created by USER-cloning using the following protocol. One µl of linearized and nicked parent vector was mixed with 1 µl of promoter fragment, 2 µl of gene fragment, 0.5 µl Taq polymerase buffer, 0.5 µl USER enzyme (NEB). The mix was incubated at 37°C for 25 min, at 25°C for 25 min and transformed into chemically competent *E. coli* DH5alpha. The clones with correct inserts were identified by colony PCR and the plasmids were isolated from overnight *E. coli* cultures and confirmed by sequencing. The generated vectors are listed in Table 4.

Table 4. Integrative expression vectors for *S. cerevisiae*

Name	Parent plasmid (Jensen *et al.*, 2014) and (Stovicek *et al., 2015)*	Cloned gene fragments	Promoters	Terminators
pCfB826 (pX-4-LoxP-SpHIS5_Sc_Aro7_G141S -Sc_Aro4_K229L)	pCfB258 (pX-4-LoxP-SpHIS5)	Sc_Aro7_G141S<-, Sc_Aro4_K229L->	<-PTEF1-PPGK1->	ScTADH1, ScTCYC1
pCfB1964 (pX-2-LoxP-KlURA3_Fsp_Tal)	pCfB255 (pX-2-LoxP-KlURA3)	Fsp_Tal<-	<-PTEF1	ScTADH1
pCfB2368 (pXII-1-LoxP-KlLEU2 Ph_CHS _Pc_4CL)	pCfB259 (pXII-1-LoxP-KlLEU2)	Ph_CHS<-, Pc_4Cl->	<-PTEF1-PPGK1->	ScTADH1, ScTCYC1
pCfB2369 (pXII-5-LoxP-KlURA3_Ms_CHI_Ms_CHR)	pCfB261 (pXII-5-LoxP-SpHIS5)	Ms_CHI<-, Ms_CHR->	<-PTEF1-PPGK1->	ScTADH1, ScTCYC1
pCfB2370 (pXII-4-LoxP-SpHIS5_At_ FLS_At_F3H)	pCfB262 (pXII-4-LoxP-SpHIS5)	At_FLS<-, At_F3H->	<-PTEF1-PPGK1->	ScTADH1, ScTCYC1
pCfB2371 (pXII-2-LoxP-KanMX_Fa_FMO_Cr_CPR<-)	pCfB2225 (pXII-2-LoxP-KanMX)	Fa_FMO-Cr_CPR<-	<-PTEF1	ScTADH1

pCfB2484 (pXII-2-LoxP-KanMX_Ph_FMO_Cr_CPR<-)	pCfB2225 (pXII-2-LoxP-KanMX)	Ph_FMO-Cr_CPR<-	<-PTEF1	ScTADH1
pCfB_NEW1 (pXII-5-LoxP-KlURA3_Ms_CHI)	pCfB261 (pXII-5-LoxP-SpHIS5)	Ms_CHI<-	<-PTEF1	ScTADH1
pCfB_NEW2 (pX-2-LoxP-KlURA3-PAL2<-pTEF1-pPGK1->C4H)	pCfB255 (pX-2-LoxP-KlURA3)	AtPAL2<-, AtC4H->	<-PTEF1-PPGK1->	ScTADH1
pCfB_NEW3 (pX-3-LoxP-KlLEU2-CYB5<-pTEF1-pPGK1->AtATR2)	pCfB257 (pX-3-LoxP-KlLEU2)	CYB5<-, AtATR2->	<-PTEF1-PPGK1->	ScTADH1
pCfB3437 (XI-5-loxP-amdSYM Ph_CHS-Pc_4CL)	pCfB2399 (XI-5-loxP-amdSYMsyn)	Ph_CHS<-, Pc_4Cl->	<-PTEF1-PPGK1->	ScTADH1, ScTCYC1
pCfB2879 (XII-1-loxP-NatMX Ms_CHI - Ms_CHR)	pCfB2197 (pXII-1-loxP-NatMXsyn)	Ms_CHI<-, Ms_CHR->	<-PTEF1-PPGK1->	ScTADH1, ScTCYC1
pCfB3643(XII-2-loxP-KanMX At_FLS - At_F3H)	pCfB2225 (pXII-2-loxP-KanMXsyn)	At_FLS<-, At_F3H->	<-PTEF1-PPGK1->	ScTADH1, ScTCYC1
pCfB3654 (XII-5-loxP-HPHMX Ph_F3H-Cr_CPR<-)	pCfB2337 (XII-5-loxP-HPHMXsyn)	F3H_PH-Cr_CPR<-	<-PTEF1	ScTADH1
pCfB3655 (XII-5-loxP-HPHMX FxAthill_F3H-Cr_CPR<-)	pCfB2337 (XII-5-loxP-HPHMXsyn)	FxAthill_F3H-Cr_CPR<-	<-PTEF1	ScTADH1
pCfB4751 (pX-2-loxP-KlURA3 AtPAL2-AtC4H)	pCfB255 (pX-2-loxP-KlURA3)	AtPAL2<-, AtC4H->	<-PTEF1-PPGK1->	ScTADH1, ScTCYC1
pCfB4752 (pX-3-loxP-KlLEU2 cyb5-AtATR2)	pCfB257 (pX-3-loxP-KlLEU2)	CYB5<-, AtATR2->	<-PTEF1-PPGK1->	ScTADH1, ScTCYC1

The integrative expression vectors were linearized with NotI, purified from the reaction mix using NucleoSpin® Gel and PCR Clean-up kit (Macherey-Nagel) and transformed into *S. cerevisiae* cells using the lithium acetate transformation protocol (Gietz *et al.*, 2002).

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Table 5. Overview of genetically modified microorganism strains

Strain/plasmid	Description

***E. coli* strains**	
DH5α	General cloning host
BL21(DE3)	*ompT hsdT hsdS* (r_B^- m_B^-) *gal* (DE3)
ST1	BL21(DE3) carrying pCDF-*talRs-4cl-2Pc* and *pET-chsPh-chiMs*
ST2am	BL21(DE3) carrying pCDF-*talRs-4cl-2Pc* and *pET-chsPh-chiMs* and *pRSF-chrAm*
ST2ms	BL21(DE3) carrying pCDF-*talRs-4cl-2Pc* and *pET-chsPh-chiMs* and *pRSF-chrMs*
ST3	BL21(DE3) carrying pCDF-*f3hAt-fls-1at*
ST4at	BL21(DE3) carrying pACYC *FMOAt-cprcr*
ST4fxa1	BL21(DE3) carrying pACYC *FMOfxa1-cprcr*
ST4fxa2	BL21(DE3) carrying pACYC *FMOfxa2-cprcr*
ST4ph	BL21(DE3) carrying pACYC *FMOph-cprcr*
ST4mxd	BL21(DE3) carrying pACYC *FMOmxd-cprcr*
ST4cr	BL21(DE3) carrying pACYC *f3'5'hcr-cprcr*
ST5at	BL21(DE3) carrying pCDF-*f3hAt-fls-1at* and pACYC *FMOat-cprcr*
ST5fxa1	BL21(DE3) carrying pCDF-*f3hAt-fls-1at* and pACYC *FMOfxa1-cprcr*
ST5fxa2	BL21(DE3) carrying pCDF-*f3hAt-fls-1at* and pACYC *FMOfxa2-cprcr*
ST5mxd	BL21(DE3) carrying pCDF-*f3hAt-fls-1at* and pACYC *FMOmxd-cprcr*
ST5cr	BL21(DE3) carrying pCDF-*f3hAt-fls-1at* and pACYC *f3'5'hcr-cprcr*
ST5ph	BL21(DE3) carrying pCDF-*f3hAt-fls-1at* and pACYC *FMOph-cprcr*

Table 6. *S. cerevisiae* strains

***S. cerevisiae* strains**	**Parent strain**	**Genetic modification**	**Phenotype**
ST_NEW1	CEN.PK102-5B	pCfB2368 (pXII-1-LoxP-KlLEU2_Pc_4CL_Ph_CHS), pCfB_NEW1 (pXII-5-LoxP-KlURA3_Ms_CHI), and pCfB2370 (pXII-4-LoxP-SpHIS5_At_F3H_At_FLS)	ura+his+leu+
ST_NEW2	CEN.PK102-5B	pCfB2368 (pXII-1-LoxP-KlLEU2_Pc_4CL_Ph_CHS), pCfB2369 (pXII-5-LoxP-KlURA3_Ms_CHI_Ms_CHR), and pCfB2370 (pXII-4-LoxP-SpHIS5_At_F3H_At_FLS)	ura+his+leu+
ST_NEW3	ST_NEW1	pCfB2371 (pXII-2-LoxP-KanMX_Fa_FMO_Cr_CPR<-)	ura+his+leu+G418^R
ST_NEW4	ST_NEW1	pCfB2484 (pXII-2-LoxP-KanMX_Ph_FMO_Cr_CPR<-)	ura+his+leu+G418^R
ST_NEW5	ST_NEW2	pCfB2371 (pXII-2-LoxP-KanMX_Fa_FMO_Cr_CPR<-)	ura+his+leu+G418^R
ST_NEW6	ST_NEW2	pCfB2484 (pXII-2-LoxP-KanMX_Ph_FMO_Cr_CPR<-)	ura+his+leu+G418^R
ST_NEW7	CEN.PK102-5B	pCfB1964 (pX-2-LoxP-KlURA3_Fsp_Tal)	ura+his-leu-
ST_NEW8	CEN.PK102-5B	pCfB_NEW2 (pX-2-LoxP-KlURA3-PAL2<-pTEF1-pPGK1->C4H) and pCfB_NEW3 (pX-3-LoxP-KlLEU2-CYB5<-pTEF1-pPGK1->AtATR2)	ura+his-leu+

ST_NEW9	ST_NEW7	pCfB826 (pX-4-LoxP-SpHIS5_Sc_Aro7_G141S-Sc_Aro4_K229L)	ura+his+leu-
ST_NEW10	ST_NEW8	pCfB826 (pX-4-LoxP-SpHIS5_Sc_Aro7_G141S-Sc_Aro4_K229L)	ura+his+leu+
ST_NEW11	ST_NEW9	Removal of selection markers	ura-his-leu-
ST_NEW12	ST_NEW10	Removal of selection markers	ura-his-leu-
ST_NEW13	ST_NEW11	pCfB2368 (pXII-1-LoxP-KlLEU2_Pc_4CL_Ph_CHS), pCfB_NEW1 (pXII-5-LoxP-KlURA3_Ms_CHI), and pCfB2370 (pXII-4-LoxP-SpHIS5_At_F3H_At_FLS)	ura+his+leu+
ST_NEW14	ST_NEW11	pCfB2368 (pXII-1-LoxP-KlLEU2_Pc_4CL_Ph_CHS), pCfB2369 (pXII-5-LoxP-KlURA3_Ms_CHI_Ms_CHR), and pCfB2370 (pXII-4-LoxP-SpHIS5_At_F3H_At_FLS)	ura+his+leu+
ST_NEW15	ST_NEW13	pCfB2371 (pXII-2-LoxP-KanMX_Fa_FMO_Cr_CPR<-)	ura+his+leu+G418^R
ST_NEW16	ST_NEW13	pCfB2484 (pXII-2-LoxP-KanMX_Ph_FMO_Cr_CPR<-)	ura+his+leu+G418^R
ST_NEW17	ST_NEW14	pCfB2371 (pXII-2-LoxP-KanMX_Fa_FMO_Cr_CPR<-)	ura+his+leu+G418^R
ST_NEW18	ST_NEW14	pCfB2484 (pXII-2-LoxP-KanMX_Ph_FMO_Cr_CPR<-)	ura+his+leu+G418^R
ST_NEW19	ST_NEW12	pCfB2368 (pXII-1-LoxP-KlLEU2_Pc_4CL_Ph_CHS), pCfB_NEW1 (pXII-5-LoxP-	ura+his+leu+

		KlURA3_Ms_CHI), and pCfB2370 (pXII-4-LoxP-SpHIS5_At_F3H_At_FLS)	
ST_NEW20	ST_NEW12	pCfB2368 (pXII-1-LoxP-KlLEU2_Pc_4CL_Ph_CHS), pCfB2369 (pXII-5-LoxP-KlURA3_Ms_CHI_Ms_CHR), and pCfB2370 (pXII-4-LoxP-SpHIS5_At_F3H_At_FLS)	ura+his+leu+
ST_NEW21	ST_NEW19	pCfB2371 (pXII-2-LoxP-KanMX_Fa_FMO_Cr_CPR<-)	ura+his+leu+G418^R
ST_NEW22	ST_NEW19	pCfB2484 (pXII-2-LoxP-KanMX_Ph_FMO_Cr_CPR<-)	ura+his+leu+G418^R
ST_NEW23	ST_NEW20	pCfB2371 (pXII-2-LoxP-KanMX_Fa_FMO_Cr_CPR<-)	ura+his+leu+G418^R
ST_NEW24	ST_NEW20	pCfB2484 (pXII-2-LoxP-KanMX_Ph_FMO_Cr_CPR<-)	ura+his+leu+G418^R
ST691 (Rodriguez *et al.*, 2015)	CEN.PK102-5B	Δaro10 Δpdc5	ura-his-leu-
ST_4314	ST691	pCfB1964 (pX-2-LoxP-KlURA3 Fsp_Tal), pCfB826 (pX-4-LoxP-SpHIS5_Sc_Aro7_G141S-Sc_Aro4_K229L), pCfB257 (pX-3-loxP-KlLEU2) pCfB3437 (XI-5-loxP-amdSYMsyn Ph_CHS- Pc_4CL), pCfB2879 (XII-1-loxP-NatMX Ms_CHI - Ms_CHR), pCfB3643(XII-2-loxP-KanMX At_FLS - At_F3H) and pCfB03655 (XII-5-loxP-HPHMXsyn FxAthill_F3H-Cr_CPR<-)	ura+his+leu+ G418^R, Hyg^R, amdSYM, Nat^R

ST_NEW25	St_4314	Isolate of a genetic cross: aro10 Δpdc5 pCfB3437 (XI-5-loxP-amdSYMsyn Ph_CHS- Pc_4CL), pCfB2879 (XII-1-loxP-NatMXsyn3 Ms_CHI - Ms_CHR), pCfB3643(XII-2-loxP-KanMXsyn At_FLS - At_F3H) and pCfB03655 (XII-5-loxP-HPHMXsyn FxAthill_F3H-Cr_CPR<-)	$G418^R$, Hyg^R, amdSYM, Nat^R
ST_NEW26	ST_NEW25	pCfB4751 (pX-2-loxP-KlURA3 AtPAL2-AtC4H), pCfB4752 (pX-3-loxP-KlLEU2 cyb5-AtATR2) and pCfB826 (pX-4-LoxP-SpHIS5_Sc_Aro7_G141S-Sc_Aro4_K229L)	ura+his+leu+ $G418^R$, Hyg^R, amdSYM, Nat^R

Example 5. Flavonoid analysis and quantification

The production of flavonoids in *E. coli* recombinant strains was analyzed and quantified using an high-performance liquid chromatograph (HPLC) equipped with a Discovery® HS F5-5 column (4.6 by 150 mm; 5.0-µm particle size; Sigma-Aldrich) connected to a UV detector (277, 290, 333 and 370 nm). Depending on the flavonoid analyzed two different methods (A) or (B) were utilized. A) A flow rate of 1.5 ml/min was used with a linear gradient of 10 mM ammonium formate pH 3.0 buffer (phase A) and acetonitrile (phase B) by the following method: 0 to 0.5 min (5% B), 0.5 to 7 min (5 to 60% B), 7 to 9.5 min (60% B), 9.5 to 9.6 min (60 to 5% B), and 9.6 to 12 min (5% B). Under these conditions, L-tyrosine was detected at retention times of 2.5 min (277 nm), *p*-coumaric acid at 5.6 min (333 nm), liquiritigenin at 6.8 min (277 nm), naringenin at 7.5 min (290 nm) and isoliquiritigenin at 8.0 min (370 nm) , respectively. Authentic L-tyrosine, *p*-coumaric acid, liquiritigenin, naringenin and isoliquiritigenin were used as standards. Calibration curves of authentic *p*-coumaric acid, naringenin, liquiritigenin and isoliquiritigenin were used for quantification. B) A flow rate of 1 ml/min was used with a linear gradient of 10 mM ammonium formate pH 3.0 buffer (phase A) and acetonitrile (phase B) by the following method: 0 to 2 min (20% B), 2 to 20 min (20 to 45% B), 20 to 22 min (45% to 20% B), and 22 to 24 min (20% B). Under these conditions, dihydrokaempferol was detected at a retention time of 10.0 min (333 nm), fisetin at 10.3 min (370 nm), liquiritigenin at 12.8 min

(277 nm), resokaempferol at 13.3 min (370 nm), quercetin at 13.9 min (370 nm), naringenin at 16.7 min (290 nm), kaempferol at 17.2 min (370 nm) and isoliquiritigenin 19.9 min (370 nm), respectively. Authentic dihydrokaempferol, fisetin, liquiritigenin, resokaempferol, quercetin, naringenin, kaempferol and isoliquiritigenin were used as standards. Calibration curves of authentic resokaempferol and authentic fisetin were used for quantification.

Authentic standards of p-coumaric acid, naringenin, isoliquiritigenin, kaempferol, dihydrokaempferol, quercetin and fisetin were purchased from Sigma-Aldrich Co. (Denmark). Furthermore, authentic standards of naringenin, chalcone, liquiritigenin and resokaempferol were acquired from MicroCombiChem e.K. (Germany), Tocris Bioscience (United Kingdom) and Extrasynthese (France), respectively.

Example 6. Biosynthesis of liquiritigenin from L-tyrosine using *E. coli*

Liquiritigenin production was carried out as follows, *E. coli* BL21(DE3) strain ST1, ST2am and ST2ms harbouring recombinant plasmids was precultured in 3 ml of 2xYT liquid medium with appropriate antibiotics (50 µg/ml streptomycin/spectinomycin for pCDFDuet; 100 µg/ml ampicillin for pETDuet; 35µg/ml kanamycin for pRSFDuet) and incubated at 37°C and 250 rpm overnight. The following day, the preculture was transferred into 2 ml of M9 minimal media (0.2% glucose), with appropriate antibiotics, to a final concentration of OD_{600} 0.05 and cultured at 37 °C and 300 rpm in 24 deep well plates (Enzyscreen B.V., Netherlands) until OD_{600} reached ~ 0.6. Then, isopropyl β-D-1 thiogalactopyranoside (IPTG) was added at a final concentration of 1 mM, and the cells were grown for 3 hours at 30°C, followed by addition of 2 mM L-tyrosine and incubation at 30°C for 24 h. Collected samples were harvested by centrifugation at 13.000 rpm (16.200 X *g*) for 10 min and the supernatant was filtered through 0.2 um filters and analyzed by HPLC.
ST1 (pCDFTALrs4CLpc and pETCHSphCHIms), which lacks the gene encoding CHR, when supplemented with 2mM L-tyrosine only produced p-coumaric acid and a small amount of naringenin after 24 hours.

ST2am (pCDFTALrs4CL2pc and pETCHSphCHIms and pRSFCHRam), supplemented with 2mM L-tyrosine produced 0.60 µM (154 µg/L) liquiritigenin after 24 hours.
ST2ms (pCDFTALrs4CL2pc and pETCHSphCHIms and pRSFCHRms), supplemented with 2mM L-tyrosine produced 0.44 µM (113 µg/L) liquiritigenin after 24 hours.
Experiments were carried out in triplicates, and the final data is shown in Table 7.

Table 7. The production of p-coumaric acid (PCA), naringenin (NRN), liquiritigenin (LIQ) and isoliquiritigenin (ILQ) using recombinant E. coli strains ST2am, ST2ms and ST1. ND denotes not detected.

	Production (µM)			
	PCA	**NRN**	**LIQ**	**ILQ**
ST2am	327,2 ± 8,0	1,36 ± 0,12	0,60 ± 0,11	0,082 ± 0,005
ST2ms	331,2 ± 6,2	1,48 ± 0,44	0,44 ± 0,04	0,063 ± 0,008
ST1	315,4 ± 6,1	3,70 ± 0,68	ND	ND

Example 7. Biosynthesis and purification of garbanzol and resokaempferol from liquiritigenin using *E. coli*

Garbanzol and resokeampferol production was demonstrated using *E. coli* BL21(DE3) harbouring pCDFF3HatFLSat (*E. coli* strain ST3), which was precultured in 3 ml of 2xYT liquid medium with appropriate antibiotics (50 µg/ml streptomycin/spectinomycin for pCDFDuet) and incubated at 37°C and 250 rpm overnight. The following day, the preculture was transferred into 2 ml of M9 minimal media (1% glucose), with appropriate antibiotics, to a final concentration of OD_{600} 0.05 and cultured at 37 °C.

The following day, the preculture was transferred into 20 ml of M9 minimal medium (0.2% glucose) with antibiotics, to a final concentration of OD_{600} 0.05 and cultured at 37°C and 300 rpm until OD_{600} of ~ 0.6 was reached. Then IPTG was added to a final concentration of 1 mM and the culture was incubated for 5 hours to increase biomass. The cell pellet was collected by centrifugation and resuspended in 100 ml of M9 minimal media (0.2% glucose) supplemented with 1 mM of IPTG and 0.1 mM liquiritigenin and cultured at 30°C and 300 rpm. The extraction of putative garbanzol and putative resokaempferol was carried out as previously described by Malla et al, 2013. Briefly, the culture was extracted with an equal volume of ethyl acetate and the organic layer was collected followed by evaporation of excess solvent to dryness. The remaining products were dissolved in 99% EtOH for HPLC and HPLC fraction collection analysis. No authentic garbanzol could be obtained to be used as a standard compound.

E. coli strain ST3, 24 hours after supplementing the culture with liquiritigenin (figure 2A) produced liquiritigenin, detected at a retention time of 12.8 min, and isoliquiritigenin at a retention time of 19.9 min. As expected, resokaempferol was detected at a retention time of

13.3, confirmed by UV spectra and retention time comparison with an authentic resokaempferol standard. An unknown peak appeared after 8.0 min (figure A) thought to be garbanzol (named in figure 2A) based on the high similarity in the UV spectrums of liquiritigenin and putative garbanzol (figure 2C). No peaks similar to putative garbanzol and resokaempferol were detected in a control experiment using BL21(DE3) carrying pCDFDuet-1 (figure 2B). Isoliquiritigenin was also detected in the control experiment as liquiritigenin can, based on pH and temperature, be non-enzymatically isomerized into Isoliquiritigenin (Simmler et al., 2013). The UV-spectra of all the compounds are shown in figure 2C.

HPLC fraction collection: Putative garbanzol and resokaempferol peaks were purified using an HPLC fraction collector equipped with a Discovery® HS F5-5 column (4.6 by 150 mm; 5.0-µm particle size; Sigma-Aldrich) connected to a UV detector (277, 290, 333 and 370 nm). A flow rate of 1ml/min was used with a linear gradient of 10 mM ammonium formate pH 3.0 buffer (phase A) and acetonitrile (phase B) by the following method: 0 to 3 min (20% B), 3 to 21 min (20 to 45% B), 21 to 23 min (45%B), 23 to 25 min (45% to 20% B) and 25 min to 33 min (20% B).

After purification, the putative garbanzol and resokaempferol were injected into an Orbitrap Fusion™ for further elucidation. The spectra for putative garbanzol infusion (Figure 3A) shows an ion with m/z 273.07565, which corresponds to within -0.4 ppm of the mass of an ion with the ionic formula of $C_{15}H_{13}O_5$ in positive ion mode; and an ion with m/z 271.06055 corresponding to within -2.4 ppm of the mass of an ion with the ionic formula of $C_{15}H_{11}O_5$ in negative ion mode, indicative of a compound with a molecular formula of $C_{15}H_{12}O_5$, which is consistent with the isolated molecule being garbanzol. Infusion of the purified resokaempferol gave the spectra (Figure 3B) which shows an ion with m/z 271.06005 corresponding to within -0.2 ppm of an ion with the ionic formula of $C_{15}H_{11}O_5$ in positive ion mode, and an ion with m/z 269.04521 corresponding to within -1.3 ppm of ion with the ionic formula of $C_{15}H_9O_5$ in positive ion mode, indicative of a compound with a molecular formula of $C_{15}H_{10}O_5$. Infusion of a resokaempferol standard (Figure 3C) gave ions with m/z 271.06010 corresponding to within 0.0 ppm of ion with the ionic formula of $C_{15}H_{11}O_5$ in positive ion mode and m/z 269.04521corresponding to within -1.4 ppm of ion with the ionic formula of $C_{15}H_9O_5$ in positive ion mode, indicative of compound with a molecular formula of $C_{15}H_{12}O_5$. Examinations of the ms^2 spectra show that the fragmentation patterns in both positive and negative ionization modes are consistent with both spectra being from resokaempferol.

Example 8. Biosynthesis of fisetin from resokaempferol using *E. coli*

Fisetin production was carried out as follows: *E. coli* BL21(DE3) harbouring recombinant plasmids was precultured in 3 ml of 2xYT liquid medium with appropriate antibiotics (25 µg/ml chloramphenicol for pAYCDuet) and incubated at 37°C and 250 rpm overnight. The following day, the preculture was transferred into 2 ml of M9 minimal media (0.2% glucose), with appropriate antibiotics, to a final concentration of OD_{600} 0.05 and cultured at 37 °C and 300 rpm in 24 deep well plates (Enzyscreen B.V., Netherlands) until OD_{600} reached ~ 0.6. Then, isopropyl β-D-1 thiogalactopyranoside (IPTG) was added at a final concentration of 1 mM, the cells grown for 3 hours at 30°C, followed by addition of the substrate, 0,1 mM resokaempferol and incubation at 30°C for 40 h. Samples were collected after 3, 24 and 40 hours. Resokaempferol and fisetin were extracted directly from 750 µl culture by adding an equal volume of 99% ethanol (EtOH) followed by vigorous shaking for one hour at room temperature. After centrifugation at 13.000 rpm (16.200 X *g*) for 10 min the supernatant was filtered through 0.2 um filters and injected into HPLC for analysis. *E. coli* strain - ST4at (pACYCFMOat) supplemented with 0,05 mM resokaempferol produced 4,1 µM (1,2 mg/L) fisetin after 3 hours, 7,2 µM (2,1 mg/L) fisetin after 18 hours and 0,2 µM (57 µg/L) after 40 hours; *E. coli* strain - ST4ph (pACYCFMOph) supplemented with 0,05 mM resokaempferol produced 0,87 µM (249 µg/L) fisetin after 3 hours, 3,2 µM (915 µg/L) fisetin after 18 hours and 0,53 µM (151 µg/L) after 40 hour and *E. coli* strain - ST4cr (pACYCF3'5'Hcr) supplemented with 0,05 mM resokaempferol produced 0,13 µM (77 µg/L) fisetin after 3 hours, 0,2 µM (57 µg/L) fisetin after 18 hours and 0,47 µM (134 µg/L) after 40 hour, as shown in figure 4A.

Experiments were carried out in triplicates.

Subsequently, in order to verify that fisetin indeed was produced, a sample collected from ST4cr was evaluated further. The HPLC detected peak, which eluted at the same retention time as the fisetin standard, was analyzed on a linear ion trap and therefore the masses can only be reported at unit mass (Figure 5A), the observed ions 287 in positive mode and 285 are consistent with those observed in the fisetin standard. The spectra for the infusion of the fisetin standard (Figure 5B) gave an ion with m/z 287.05487 which corresponds to within -0.5 ppm of ion with the ionic formula of $C_{15}H_{11}O_6$ in positive ion mode and an ion with m/z 285.03997 corresponding to within -1.7 ppm of ion with the ionic formula of $C_{15}H_9O_6$ in positive ion mode, indicative of compound with a molecular formula of $C_{15}H_{10}O_6$. The ms^2 spectra show that the fragmentation patterns in both positive and negative ionization modes are consistent with both spectra being from fisetin (figure 5).

Example 9. Biosynthesis of fisetin from liquiritigenin using *E. coli*

Fisetin production was demonstrated as follows, *E. coli* BL21(DE3) strains ST5at, ST5ph and ST5cr harbouring recombinant plasmids were precultured in 3 ml of 2xYT liquid medium

with appropriate antibiotics (50 µg/ml streptomycin/spectinomycin for pCDFDuet; 25 µg/ml chloramphenicol for pAYCDuet) and incubated at 37°C and 250 rpm overnight. The following day, the preculture was transferred into 2 ml of M9 minimal media (0.2% glucose), with appropriate antibiotics, to a final concentration of OD_{600} 0.05 and cultured at 37 °C and 300 rpm in 24 deep well plates (Enzyscreen B.V., Netherlands) until OD_{600} reached ~ 0.6. Then, isopropyl β-D-1 thiogalactopyranoside (IPTG) was added at a final concentration of 1 mM, the cells grown for 3 hours at 30°C, followed by addition of the substrate, 0.1 mM liquiritigenin and incubation at 30°C for 18 h. Samples were collected after 3, 6 and 18 hours. Garbanzol, resokaempferol and fisetin were extracted directly from 750 µl culture by adding an equal volume of 99% ethanol (EtOH) followed by vigorous shaking for one hour at room temperature. After centrifugation at 13.000 rpm (16.200 X *g*) for 10 min the supernatant was filtered through 0.2 um filters and injected into HPLC for analysis. *E. coli* strain-ST5at (pCDFF3HatFLSat and pACYCFMOat), supplemented with 0,05 mM liquiritigenin produced 1,3 µM (372 µg/L) fisetin after 3 hours, 0,7 µM (200 µg/L) fisetin after 6 hours and 0 µM after 18 hours; *E. coli* strain-ST5ph (pCDFF3HatFLSat and pACYCFMOph) supplemented with 0,05 mM liquiritigenin produced 0,5 µM (143 µg/L) fisetin after 3 hours, 0,3 µM (86 µg/L) fisetin after 6 hours and 0,2 µM (57 µg/L) after 18 hours and *E. coli* strain-ST5cr (pCDFF3HatFLSat and pACYCF3'5'Hcr) and supplemented with 0,05 mM liquiritigenin produced 0 µM fisetin after 3, 6, 18 and 24hours.
Experiments were carried out in triplicates (see figure 4B, C).

Example 10. Production of resokaempferol from p-coumaric acid in *S. cerevisiae*

S. cerevisiae CEN.PK102-5B (ura-his-leu-) was transformed with vectors pCfB2368 (pXII-1-LoxP-KlLEU2_Pc_4CL_Ph_CHS), pCfB2369 (pXII-5-LoxP-KlURA3_Ms_CHI_Ms_CHR), and pCfB2370 (pXII-4-LoxP-SpHIS5_At_F3H_At_FLS) and the transformants were selected on SC drop-out medium without histidine, uracil and leucine. The resulting strain ST_NEW2 (Table 6) is cultivated in synthetic fed-batch medium supplemented with 0.4 g/L p-coumaric acid in order to obtain resokaempferol.

Example 11. Production of fisetin from p-coumaric acid in *S. cerevisiae*

The strain ST_NEW2 was transformed with vector pCfB2371 (pXII-2-LoxP-KanMX_Fa_FMO_Cr_CPR<-) or pCfB2484 (pXII-2-LoxP-KanMX_Ph_FMO_Cr_CPR<-) and the transformants were selected on SC medium supplemented with 200 mg/L G418 (the ammonium sulphate in the medium was replaced with sodium monoglutamate). The resulting strains ST_NEW5 and ST_NEW6 (Table 6), expressing correspondingly Fa_FMO or Ph_FMO, are cultivated in synthetic fed-batch medium supplemented with 0.4 g/L p-coumaric acid in order to obtain fisetin.

Example 12. Production of p-coumaric acid from sugars in *S. cerevisiae*

S. cerevisiae CEN.PK102-5B (ura-his-leu-) was transformed with vector pCfB1964 (pX-2-LoxP-KlURA3_Fsp_Tal) and selected on SC drop-out plates without uracil to give strain ST_NEW7 (Table 6). *S. cerevisiae* CEN.PK102-5B (ura-his-leu-) was transformed with vectors pCfB_NEW2 (pX-2-LoxP-KlURA3-PAL2<-pTEF1-pPGK1->C4H) and pCfB_NEW3 (pX-3-LoxP-KlLEU2-CYB5<-pTEF1-pPGK1->AtATR2) and selected on SC drop-out plates without uracil and leucine to give strain ST_NEW8 (Table 6). The strains ST_NEW7 and ST_NEW8 were cultivated in synthetic fed-batch medium with or without supplementation with 5 mM tyrosine or 5 mM phenylalanine to obtain p-coumaric acid.

In order to further improve the production of p-coumaric acid directly from sugars, the flux towards tyrosine and phenylalanine biosynthesis was improved by overexpressing a feedback-inhibition resistant allele of 3-deoxy-D-arabino-heptulosonate-7- phosphate synthase $ARO4^{K229L}$ and a feedback-inhibition resistant allele of chorismate mutase $ARO7^{G141S}$ from *S. cerevisiae.* This was done as following: ST_NEW7 strain was transformed with pCfB826 (pX-4-LoxP-SpHIS5_Sc_Aro7_G141S-Sc_Aro4_K229L) to result in strain ST_NEW9, which was selected on SC drop-out plates without uracil and histidine (Table 6). ST_NEW8 strain was transformed with pCfB826 (pX-4-LoxP-SpHIS5_Sc_Aro7_G141S-Sc_Aro4_K229L) to result in strain ST_NEW10, which was selected on SC drop-out plates without uracil and histidine (Table 6). The production of p-coumaric acid is assessed in ST_NEW9 and ST_NEW10 in synthetic fed-batch medium without supplementation of aromatic amino acids.

Example 13. Production of resokempferol, and fisetin from sugars in *S. cerevisiae*

The SpHIS5, KlURA3, and KlLEU2 markers were removed from the strains ST_NEW9 and ST_NEW10, using CreA-mediated recombination as described previously (Jensen *et al.*, 2014), giving correspondingly strains ST_NEW11 and ST_NEW12 (Table 6). The transformations to obtain strains producing resokaempferol or fisetin were performed according to Table 4. The production of the products by strains ST_NEW13-24 (Table 6) was assessed on synthetic fed-batch medium.

At least six independent yeast transformants of strains ST_NEW17 and ST_NEW18 (Table 6) were inoculated in 0.5 ml SC ura-his-leu- medium with 200 µg/mL of G418 disulfate salt in a 96-deep-well microtiter plate with air-penetrable lid (EnzyScreen, Germany). The plates were incubated at 30°C with 250 rpm agitation at 5 cm orbit cast overnight. 50 µl of the overnight cultures were used to inoculate 0.5 ml production medium in a new 96-deep well plate (EnzyScreen, Germany). The production medium was synthetic fed-batch medium for

S. cerevisiae M-Sc.syn-1000 (M2P labs GmbH, Germany). The medium was prepared by supplementing it with the supplied vitamins solution (final 1% v/v) and the enzyme mix (final concentration 0.5% v/v) immediately prior to use. The medium contains a soluble sugar polymer, which slowly releases glucose under the action of the added enzyme. This glucose comprises the only carbon source in the medium. Fermentation was carried out for 72 hours at the same conditions as above. The culture broth was spun down and the supernatants were analysed for fisetin concentration using HPLC. The concentration of fisetin in the fermentation broth of ST_NEW17 and ST_NEW18 strains was 0.33±0.02 mg/L and 0.36±0.04 mg/L respectively.

The identity of fisetin in the samples was confirmed by LC-MS analysis (Figure 6).

Example 14 Construction of recombinant plasmids for functional expression of a fisetin pathway in *Lactococcus lactis*

The following genes will be cloned for the functional expression of a fisetin pathway in *Lactococcus lactis*: tyrosine ammonia lyase from *Flavobacterium johnsoniae* (TAL^{Fj}; gi 146298870), 4-coumaroyl-CoA ligase from *Petroselinum crispum* ($4CL^{Pc}$; gi 112801), chalcone synthase from *Petunia hybrida* (CHS^{Ph}; gi 7331152), chalcone isomerase *Medicago sativa* (CHI^{Ms}; gi 116134), 6'-deoxychalcone synthase from *Astragalus mongholicus* (CHR^{Am}; gi 302129635), flavanone 3-hydroxylase from *Arabidopsis thaliana* ($F3H^{At}$; gi 15230433), flavonol synthase from *Arabidopsis thaliana* (FLS^{At}; gi 334187530), fusion of *Arabidopsis thaliana* flavonoid 3'-monooxygenase (FMO^{At}; gi 15241483) and *Catharanthus roseus* cytochrome P450 reductase (CPR; gi 730125). Primer sequences for amplification of gene fragments are listed in Table 8. The gene fragments as well as primers and templates used for their PCR amplification are listed in Table 9. *L. lactis* NZ9000 is used as a cloning host for intermediate and final expression plasmids (Table 10).

a) Construction of a recombinant plasmid for functional expression of a L-tyrosine to liquiritigenin pathway in *L. lactis*

The TAL_Fj fragment (Table 9) will be cloned into pNZ8048 at *Nco*I/*Sph*I restriction sites to yield pNZ_TAL^{Fj} (1) plasmid. The 4CL_Pc fragment, containing the $4CL^{Pc}$ gene transcriptional fused to P_{nisA} promoter (Table 9), will be cloned into pNZ_TAL^{Fj} (1) at *Sph*I/*Spe*I restriction sites to originate plasmid pNZ_TAL^{Fj}_$4CL^{Pc}$. The PCR fragment CHS^{Ph}_CHI^{Ms} (Table 9), containing the CHS^{Ph} gene transcriptional fused to P_{nisA} promoter and CHI^{Am} gene preceded by a consensus lactococcal ribosome binding site (5' TAAAGGAGG 3') and a 7-base spacer (5' AATAATA 3') (de Vos, 1987) at ATG initiation codon, will be amplified from pNZ_CHS^{Ph}_CHI^{Ms} and further introduced into pNZ_TAL^{Fj}_$4CL^{Pc}$ at *Spe*I/*Xba*I restriction sites

to create plasmid pNZ_TAL^{Fj}_4CL^{Pc}_CHS^{Ph}_CHI^{Ms}. Finally, the expression plasmid containing a complete heterologous pathway to produce liquiritigenin from L-tyrosine will be constructed by cloning the CHR_Am fragment (P_{nisA} promoter transcriptional fused to CHR^{Am} gene (Table 3)) into *Xba*I restriction site of pNZ_TAL^{Fj}_4CL^{Pc}_CHS^{Ph}_CHI^{Ms}, originating pNZ_TAL^{Fj}_4CL^{Pc}_CHS^{Ph}_CHI^{Ms}_CHR^{Am}. Liquiritigenin production will be tested by expressing pNZ_TAL^{Fj}_4CL^{Pc}_CHS^{Ph}_CHI^{Ms}_CHR^{Am} into NZ9000Δ*ldh*Δ*ldhB* (strain *L. lactis* ST1).

b) Construction of a recombinant plasmid for functional expression of an enzymatic pathway for the conversion of liquiritigenin to fisetin in *L. lactis*

The F3H_At fragment (Table 9) will be cloned into pILZ_Em at *Nco*I/*Kpn*I restriction sites to yield pILZ_F3H^{At} plasmid. The FLS_At fragment (Table 9), containing the P_{czcD} promoter transcriptional fused to FLS^{At} gene, will be cloned into pILZ_F3H^{At} at *Kpn*I/SpeI restriction sites to originate plasmid pILZ_F3H^{At}_FLS^{At}. The FMO_At_CPR_Cr fragment (Table 9) containing the P_{czcD} promoter transcriptional fused to a gene fusion of FMO^{At} and CPR^{Cr}, will be introduced into pILZ_F3H^{At}_FLS^{At} at *Spe*I/*Sac*I restriction sites to create plasmid pILZ_F3H^{At}_FLS^{At}_FMO^{At}_CPR^{Cr}. The latter plasmid contains a complete heterologous pathway to produce fisetin from liquiritigenin. Production will be tested by expressing pILZ_F3H^{At}_FLS^{At}_FMO^{At}_CPR^{Cr} into strain *L. lactis* ST1 (strain *L. lactis* ST2).

C) Biosynthesis of liquiritigenin and fisetin from L-tyrosine using *L. lactis*

Production of liquiritigenin and fisetin ise assessed as follows, strains *L. lactis* ST1 or ST2 carrying the recombinant plasmids is precultured in 5 ml of chemically defined medium (CDM; Poolman and Konings 1988) supplemented with 0.2% glucose (W/V) and 5 µg/ml of the appropriate antibiotics, and incubated overnight at 30°C. In the following day, the preculture is transferred into 25 ml of CDM (0.2% glucose), with appropriate antibiotics, to a final OD_{600} 0.05 and cultured at 30 °C in 50 mL screw-cap tubes until OD_{600} reached ~ 0.3. At this point gene expression is induced by adding nisin (final concentration of 1.5 ng/mL) alone or in combination with 0.7 mM $ZnSO_4$ to *L. lactis* ST1 and ST2 cultures, respectively. At distinct time points culture samples are harvested by centrifugation at 13.000 rpm (16.200 X *g*) for 10 min at 4°C and the supernatant kept at -20°C until further analysis by HPLC and/or LC-MS.

Table 8. Primers for reconstruction of fisetin pathway in *L. lactis*

Primer name	Primer sequence, 5'→3'	*Restriction site*	SEQ ID No
TAL_Fj_fw1	CATGTCATGAACACCATCAACGAATATC	*BspHI*	231
TAL_Fj_rv2	CAGTGCATGCTTAATTGTTAATCAGGTGGTC	*SphI*	232
4CL_fw4	CAGTGCATGCAGATCTAGTCTTATAACTATACTGAC	*SphI*	233

4CL_Pc_rv3	GGACTAGTTTATTTGGGAAGATCACCGGATGC	*SpeI*	234
PnisA_fw1	GGACTAGTAGATCTAGTCTTATAACTATACTGAC	*SpeI*	235
CHI_Ms_rv1	GCTCTAGATTAGTTGCCGATTTTAAAGGC	*XbaI*	236
PnisA_fw2	GCTCTAGAAGATCTAGTCTTATAACTATACTGAC	*XbaI*	237
CHR_Am_rv1	GCTCTAGATTAGATTTCGTCATCCCAC	*XbaI*	238
F3H_At_fw1	CATGCCATGGCTCCAGGAACTTTGAC	*NcoI*	239
F3H_At_rv1	GCGGTACCCTAAGCGAAGATTTGGTCG	*KpnI*	240
FLS_At_fw1	CATGCCATGGAGGTCGAAAGAGTCC	*NcoI*	241
FLS_At_rv1	GGACTAGTTCAATCCAGAGGAAGTTTATTG	*SpeI*	242
PczcD_fw1	GCGGTACCATGGACACTTAAGGCAAATTGTTC	*KpnI*	243
FMO_At_fw1	CATGCCATGGCGACCATTCTGCTGGC	*NcoI*	244
CPR_Cr_rv1	GATCGAGCTCTTACCAAACGTCACGCAGATAACG	*SacI*	245
PcscD_fw2	GGACTAGTATGGACACTTAAGGCAAATTGTTC	*SpeI*	246

Restriction sites are underlined in primer sequences.

Table 9. Gene fragments to be amplified by PCR

DNA fragment	Fragment description	Fw primer	Rv primer	Template DNA
TAL_Fj	Tyrosine ammonia lyase from Flavobacterium johnsoniae	TAL_Fj_fw1	TAL_Fj_rv2	pNZ_TAL Fj
4CL_Pc	4-coumaroyl-CoA ligase from Petroselinum crispum with PnisA promoter at 5′-terminus	4CL_fw4	4CL_Pc_rv 3	pNZ_4CL Pc
CHSPh _CHIMs	CHS_Ph preceded of PnisA promoter at 5′-terminus and CHI_Ms preceded of a L. lactis RBS consensus sequence	PnisA_fw1	CHI_Ms_rv 1	pNZ_CHS Ph_CHIM s
CHR_A m	6'-deoxychalcone synthase from Astragalus mongholicus with PnisA at 5′-terminus	PnisA_fw2	CHR_Am_r v1	pNZ_CHR Am
F3H_At	Flavanone 3-hydroxylase from Arabidopsis thaliana	F3H_At_fw 1	F3H_At_rv 1	pCDFF3H atFLSat
FLS_At	Flavonol synthase from Arabidopsis thaliana with PczcD promoter at 5′-terminus	PczcD_fw1	FLS_At_rv1	pILZ_FLS At
FMO_At _ CPR_Cr	Fusion of flavonoid 3′-monooxygenase from Arabidopsis thaliana and cytochrome P450 reductase from Catharanthus roseus with PczcD promoter at 5′-terminus of FMO	PcscD_fw2	CPR_Cr_rv 1	pILZ_FM OAtCPRCr

Table 10. *L. lactis* strains and expression vectors

Strain/plasmid	Description
L. lactis strains	
NZ9000	MG1363 carrying *pepN*::*nisRK*, used as cloning host.
NZ9000Δ*ldh*Δ*ldhB*	NZ9000-derivative containing deletions of *ldh* and *ldhB* genes (Gaspar *et al.*, manuscript in preparation), used as expression host.
*L. lactis*_ST1	NZ9000Δ*ldh*Δ*ldhB* carrying

	pNZ_TAL^{Fj}_4CL^{Pc}_CHS^{Ph}_CHI^{Ms}_CHR^{Am}
*L. lactis*_ST2	NZ9000Δ*ldh*Δ*ldhB* carrying pNZ_TAL^{Fj}_4CL^{Pc}_CHS^{Ph}_CHI^{Ms}_CHR^{Am} and pILZ_F3H^{At}_FLS^{At}_FMO^{At}_CPR^{Cr}
L. lactis plasmids	
pNZ8048	Cm^{R}; inducible expression vector carrying P_{nisA} (Kuipers *et al.*, 1998)
pILZ_Em	Ery^{R}; inducible expression vector carrying P_{czcD} (Mu *et al.*, 2013)
pNZ_TAL^{Fj} (1)	Cm^{R}; pNZ8048 carrying tyrosine ammonia lyase from *Flavobacterium johnsoniae*
pNZ_TAL^{Fj}_4CL^{Pc}	Cm^{R}; pNZ_TAL^{Fj} (1) carrying 4-coumaroyl-CoA ligase *Petroselinum crispum*
pNZ_TAL^{Fj}_4CLPc_ CHS^{Ph}_ CHI^{Ms}	Cm^{R}; pNZ_TAL^{Fj}4CL^{Pc} carrying chalcone synthase from *Petunia hybrida* and chalcone isomerase from *Medicago sativa*
pNZ_TAL^{Fj}_4CL^{Pc}_CHS^{Ph}_CHI^{Ms}_CHR^{Am}	Cm^{R}; pNZ_TAL^{Fj}_4CLPc_ CHS^{Ph}_ CHI^{Ms} carrying 6'-deoxychalcone synthase from *Astragalus mongholicus*
pILZ_F3H^{At}	Ery^{R}; pILZ_Em carrying flavanone 3-hydroxylase from *Arabidopsis thaliana*
pILZ_F3H^{At}_FLS^{At}	Ery^{R}; pILZ_F3H^{At} carrying flavonol synthase from *Arabidopsis thaliana*
pILZ_F3H^{At}_FLS^{At}_FMO^{At}_CPR^{Cr}	Ery^{R}; pILZ_F3H^{At}_FLS^{At} carrying flavonoid 3'-monooxygenase from *Arabidopsis thaliana* and cytochrome P450 reductase from *Catharanthus roseus*

Example 15 Construction of recombinant plasmids for functional expression of an enzymatic pathway for the conversion of L-tyrosine to fisetin in E. coli

pCDFTALrs4CL2pc (example 1) was linearized (digested) with restriction nuclease enzymes NcoI and SalI. Subsequently, utilizing Gibson assembly, the DNA fragment, TALrs4CL2pc, along with the T7 promoters was PCR amplified using primer pair TAL4CL_F and TAL4CL_R and cloned into pRSFDuet-1 creating pRSFTALrs4CLpc. pRSF was linearized using primer pair pRSF_F and pRSF_R (Table 11).

The gene encoding CHR from Astragalus mongholicus (CHRam, genBank accession no. HM357239) (example 1) was fused to the gene encoding CHSph (example 1), utilizing Gibson assembly, creating pETCHSphCHRamCHIms (SEQ ID No. 247) encoding the fusion polypeptide (SEQ ID No:248). The STOP codon "TAG" of CHSph and the start codon "ATG" of CHRam was replaced by a linker motif -GGGTCG. pETCHSphCHIms (example 1) was linearized using primer pair CHSfusion_F and CHSfusion_R (Table 11). CHRam was amplified using primer pair CHRfusion_F and CHRfusion_R (Table 11).

Primers	Oligonucleotide sequences (5'-3')*	SEQ ID NO
TAL4CL_F	TACAATACGATTACTTTCTGTTCGACTTAA CTCCCTTATGCGACTCCTGC	SEQ ID NO 249
TAL4CL_R	AAGCTGACGACCGGGTCTCCGCAAGTGGCA GTCGTCGGTTCAGGGCAGGG	SEQ ID NO 250
pRSF_F	AAGCTGACGACCGGGTCTCCGCAAGTGGCA	SEQ ID NO 251
pRSF_R	TTAAGTCGAACAGAAAGTAATCGTATTGTA	SEQ ID NO 252
CHSfusion_F	CTGTGGGATGACGAAATCTAAGGCTGCAGGTCGACAAGCTT	SEQ ID NO 253
CHSfusion_R	TTTTCAACGCTAACGCTACCCGACCCAGTAGCAACACTGTGGAGGACAA	SEQ ID NO 254
CHRfusion_F	TTGTCCTCCACAGTGTTGCTACTGGGTCGGGTAGCGTTAGCGTTGAAAA	SEQ ID NO 255
CHRfusion_R	CGGCCGCAAGCTTGTCGACCTGCAGCCTTAGATTTCGTCATCCCACAG	SEQ ID NO 256

Table 11. Primers for *E. coli* pathway construction

*** Restriction site: NA**

Plasmids were transformed into E. coli BL21(DE3) creating strain ST2fusion (pRSFTALrs4CLpc, pETCHSphCHRamCHIms).

Lastly all plasmids (pRSFTALrs4CLpc, pETCHSphCHRamCHIms, pCDFF3HatFLSat and pACYCFMOatCPRcr) were transformed into E. coli BL21(DE3) creating strain ST6at and (pRSFTALrs4CLpc, pETCHSphCHRamCHIms, pCDFF3HatFLSat and pACYCFMOphCPRcr) creating strain ST6ph.

Table 12. Overview of genetically modified microorganism strains

Strain/plasmid	Description
***E. coli* strains**	
ST2fusion	BL21(DE3) carrying p*RSF-talRs-4cl-2Pc; pET-chsPhchrAm-chiMs*
ST6at	BL21(DE3) carrying p*RSF-talRs-4cl-2Pc; pET-chsPhchrAm-chiMs;* pCDF-*f3hAt-fls-1at* and pACYC *FMOat-cprcr*

ST6ph	BL21(DE3) carrying p*RSF-talRs-4cl-2Pc; pET-chsPhchrAm-chiMs;* pCDF-*f3hAt-fls-1at* and pACYC *FMOph-cprcr*

Example 16. Biosynthesis of liquiritigenin from L-tyrosine using CHS-CHR fusion protein in *E. coli*

A key step in the biosynthetic pathway of fisetin from L- tyrosine is the coordinated activities of CHR and CHS. These enzymes catalyse the conversion of p-coumaroyl-CoA into isoliquiritigenin and subsequently liquiritigenin, garbanzol, resokaempferol and fisetin. The coordinated activity of CHR and CHS directs the metabolic flux of this biosynthetic pathway away from the production of naringenin chalcone and subsequently naringenin, dihydrokaempferol, kaempferol and quercetin.

Liquiritigenin production from L-tyrosine was demonstrated as follows: *E. coli* BL21(DE3) strains ST1, ST2am and ST2fusion (see Table 12), harbouring recombinant plasmids, were precultured in 3 ml of 2xYT liquid medium with appropriate antibiotics (50 µg/ml streptomycin/ spectinomycin for pCDFDuet; 100 µg/ml ampicillin for pETDuet; 35µg/ml kanamycin for pRSFDuet) and incubated at 37°C and 250 rpm overnight. The precultures were then transferred into 2 ml of M9 minimal media (0.2% glucose), with appropriate antibiotics, to a final cell density of OD600 0.05 and cultured at 37 °C and 300 rpm in 24 deep well plates (Enzyscreen B.V., Netherlands) until the OD600 reached ~ 0.6. Then, isopropyl β-D-1 thiogalactopyranoside (IPTG) was added at a final concentration of 1 mM, and the cells were grown for a further 3 hours at 30°C, followed by addition of 0.5 mM L-tyrosine and incubation at 30°C for a further 24 h. The cultured cells were then harvested by centrifugation at 13.000 rpm (16.200 X g) for 10 min and the supernatant was filtered through 0.2 um filters and analyzed by HPLC. Supplemented with 0.5 mM L-tyrosine, strain ST1 produced p-coumaric acid (62 µM), naringenin (3.0 µM) while no isoliquiritigenin and liquiritigenin was produced since the CHR enzyme, required to direct the entire pathway towards liquiritigenin was absent from this strain. Supplemented with 0.5 mM L-tyrosine, strain ST2am produced p-coumaric acid (107.5 µM), naringenin (0.82 µM), isoliquiritigenin (0.05 µM) and liquiritigenin (2.0 µM). Supplemented with 0.5 mM L-tyrosine, strain ST2fusion produced p-coumaric acid (79.4 µM), naringenin (0.39 µM), isoliquiritigenin (1.6 µM) and liquiritigenin (7.2 µM) (Table 12).

Table 13. The production of p-coumaric acid (PCA), naringenin (NRN), liquiritigenin (LIQ) and isoliquiritigenin (ILQ) using recombinant *E. coli* strains ST1, ST2am and ST2fusion.

	Production (µM)			
	PCA	**NRN**	**LIQ**	**ILQ**
ST2am	107,5 ± 1,55	0,82 ± 0,12	2,0 ± 0,29	0,05 ± 0,07
ST2fusion	79,4 ± 14,1	0,39 ± 0,05	7,2 ± 0,90	1,6 ± 0,28
ST1	62,0 ± 2,40	3,0 ± 0,29	0 ± 0,0	0 ± 0,0

The flux towards liquiritigenin was increased compared to naringenin when CHR was fused together with CHS (ST2fusion), with concentration of liquiritigenin 18-fold higher than naringenin. In contrast, when CHR was cloned alone (ST2am), the amount of liquiritigenin was only two-fold higher than naringenin. Thus, more flux was directed towards liquiritigenin, when CHR was fused together with CHS. We show that co-expression of CHS and CHR 1:1 (rather than separately) results in increased *in vivo* flux towards liquiritigenin in E. coli.

Example 17. Biosynthesis of fisetin from L-tyrosine using *E. coli*

Fisetin production from L-tyrosine was demonstrated as follows: *E. coli* BL21(DE3) strain ST6 harbouring recombinant plasmids was precultured in 3 ml of 2xYT liquid medium with appropriate antibiotics (50 µg/ml streptomycin/spectinomycin for pCDFDuet; 100 µg/ml ampicillin for pETDuet; 35µg/ml kanamycin for pRSFDuet; 25 µg/ml chloramphenicol for pAYCDuet) and incubated at 37°C and 250 rpm overnight. The preculture was then transferred into 2 ml of M9 minimal media (0.2% glucose and 2% glycerol), with appropriate antibiotics, to a final cell density of OD600 0.05 and cultured at 37 °C and 300 rpm in 24 deep well plates (Enzyscreen B.V., Netherlands) until the OD600 reached ~ 0.6. Then, isopropyl β-D-1 thiogalactopyranoside (IPTG) was added at a final concentration of 1 mM, and the cells were further grown for 3 hours at 30°C, followed by the addition of 0.5 mM L-tyrosine and further incubation at 30°C for 48 h. The cultured cells were harvested by centrifugation at 13.000 rpm (16.200 X g) for 10 min and the supernatant was filtered through 0.2 um filters and analyzed by HPLC. Experiments were carried out in triplicates. As seen in Table 14, ST6at (pRSFTALrs4CLpc, pETCHSphCHRamCHIms, pCDFF3HatFLSat and pACYCFMOatCPRcr), supplemented with 0.5 mM L-tyrosine produced 61.3µM p-coumaric acid and 0.9 µM fisetin after 48 hours. ST6ph (pRSFTALrs4CLpc, pETCHSphCHRamCHIms, pCDFF3HatFLSat and pACYCFMOphCPRcr) supplemented with 0.5 mM L-tyrosine produced 73.6µM p-coumaric acid and 1.0µM fisetin after 48 hours.

Garbanzol was detected in all samples. Accordingly, this is the first demonstration of a functional biosynthetic pathway from L-tyrosine to fisetin in a bacterium, and this establishes that *E. coli* is a suitable microbial platform strain for the production of fisetin and related flavonols.

Table 14. Production of fisetin (FIS) and detected intermediates, p-coumaric acid (PCA) and garbanzol (GAR) from L-tyrosine using recombinant *E. coli* strains ST6at and ST6ph.

	Production (µM)	
	ST6at	ST6ph
PCA	61,3 ± 6,1	73,6 ± 26,1
GAR	Detected	Detected
FIS	0,9 ± 0,2	1,0 ± 0,4

Example 18. Production of liquiritigenin, resokempferol, and fisetin from sugars in *S. cerevisiae* via L-Phenylalanine branch

There are two plausible biosynthetic routes of the fisetin production in microorganisms (see Figure 1). The fisetin biosynthesis from sugars in *S. cerevisiae* via the L-Tyrosine route was successfully demonstrated in the example 13 (see above). To confirm an alternative (via L-Phenylalanine branch) production route of fisetin in *S. cerevisiae*, the following experiments were performed.

ST_4314 strain harboring integrated plasmids pCfB1964, pCfB826, pCfB257, pCfB3437, pCfB2879, pCfB3643 and pCfB3655 (see Table 6), was sexually crossed with a CEN.PK (ura-his-leu-) strain of an opposite mating type. After sporulation and tetrad dissection, an auxotrophic strain ST_NEW25 (see Table 6) with integrated plasmids pCfB3437, pCfB2879, pCfB3643 and pCfB3655 was isolated. Lastly, the ST_NEW25 was sequentially transformed with pCfB4751 (pX-2-loxP-KlURA3 AtPAL2-AtC4H), pCfB4752 (pX-3-loxP-KlLEU2 cyb5-AtATR2) and pCfB826 (pX-4-LoxP-SpHIS5_Sc_Aro7_G141S-Sc_Aro4_K229L) to result in a final strain ST_NEW26. Transformed cells were selected on SC drop-out plates lacking uracil, histidine and leucine. For the selection of dominant markers (kanMX, hphMX, natMX and amdSYM) the SC-drop out medium was modified as follows; the ammonium sulfate was replaced by 6.6 g/l potassium sulfate, and supplemented with 200 µg/mL G418 (Sigma-

Aldrich), 200 µg/mL Hygromycin B (Sigma-Aldrich), 100 µg/mL Nourseothricin (Werner BioAgents) and 0.6 g/L of acetamide.

Fisetin, liquiritigenin and resokaempferol production was demonstrated as follows; six biological replicas of ST_NEW26 were inoculated to 500 µl of SC ura-his-leu- medium in a 96-deep-well microtiter plate with air-penetrable lid (EnzyScreen, Germany). The cultures were incubated at 30°C with 250 rpm overnight; and were then used to inoculate 3 ml of a production medium in a 24-deep well plate (EnzyScreen, Germany) at a starting OD_{600} of 0.2. The production medium was synthetic fed-batch medium for *S. cerevisiae* M-Sc.syn-1000 (M2P labs GmbH, Germany). The medium was freshly prepared by supplementing it with the supplied vitamins solution (final 1% v/v) and the enzyme mix (final concentration 0.5% v/v) prior to use. The medium contains a soluble sugar polymer, which slowly releases glucose under the action of the added enzyme and, therefore, mimics a fed-batch fermentation conditions. ST_NEW26 strains were incubated in the production medium for 72h at 30°C with constant 250 rpm shaking. At the end of the cultivation period, HPLC samples were prepared as follows; the culture broth was spun down at 16000xg for 5min and the supernatant was transferred into HPLC vials. In order to detect fisetin, liquiritigenin and resokaempferol the HPLC method described in the example 5 was implemented. The results of the HPLC analysis are depicted in Table 15 (see below).

Table 15. Production of liquiritigenin, resokempferol, and fisetin in the ST_NEW26 strain.

Compound	Titer (µM)
Liquiritigenin	7.10 ± 0.17
Resokaempferol	0.83 ± 0.06
Fisetin	0.07 ± 0.01

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Claims

1. A genetically modified micro-organism for production of fisetin; wherein said microorganism is characterised by having transgenes encoding:

   a. a polypeptide having flavanone 3-hydroxylase activity;

   b. a polypeptide having flavonol synthase activity; and

   c. a polypeptide having flavonoid 3'-monooxygenase activity; and

   wherein one of said transgenes or a native gene of said micro-organism encodes a polypeptide having the enzymatic activity of:

   d. a cytochrome P450 reductase.

2. A genetically modified micro-organism according to claim 1, wherein said microorganism is characterised by one or more additional transgene encoding one or more polypeptide having an enzymatic activity selected from the group consisting of:

   a. a chalcone isomerase;

   b. a chalcone synthase and a chalcone reductase and a chalcone isomerase;

   c. a 4-coumaroyl-CoA ligase and a chalcone synthase and a chalcone reductase and a chalcone isomerase;

   d. a tyrosine ammonia-lyase and a 4-coumaroyl-CoA ligase and a chalcone synthase and a chalcone reductase and a chalcone isomerase;

   e. a phenylalanine ammonia lyase and a 4-coumaroyl-CoA ligase and a chalcone synthase and a chalcone reductase and a chalcone isomerase; and

   f. a phenylalanine ammonia lyase and a cinnamate 4-hydroxylase and a NADPH:cytochrome P450 reductase and a 4-coumaroyl-CoA ligase and a chalcone synthase and a chalcone reductase and a chalcone isomerase.

3. A genetically modified micro-organism for production of garbanozol; wherein said microorganism comprises transgenes encoding polypeptides; wherein the polypeptides have enzymatic activities of:

a. a tyrosine ammonia-lyase, a 4-coumaroyl-CoA ligase, a chalcone synthase, a chalcone reductase, a chalcone isomerase and a flavanone 3-hydroxylase; or

b. a phenylalanine ammonia lyase, a 4-coumaroyl-CoA ligase, a chalcone synthase, a chalcone reductase, a chalcone isomerase, and a flavanone 3-hydroxylase, and optionally a cinnamate 4-hydroxylase and a NADPH:cytochrome P450 reductase.

4. A genetically modified micro-organism according to claim 3, wherein said microorganism additionally comprises a transgene encoding a polypeptide, wherein said polypeptide has flavonol synthase enzymatic activity.

5. A genetically modified micro-organism according to any one of claims 1 to 4, wherein the polypeptide having flavanone 3-hydroxylase (F3H) activity comprises an amino acid sequence having at least 80% sequence identity to an amino acid sequence selected from among any one of Arabidopsis thaliana F3H (SEQ ID NO: 2); *Capsella rubella* F3H (SEQ ID NO: 4); *Brassica napus* F3H (SEQ ID NO: 6); *Eutrema salsugineum* F3H (SEQ ID NO: 8); *Gossypium hirsutum* F3H (SEQ ID NO: 10); *Ampelopsis grossedentata* F3H (SEQ ID NO: 12); *Theobroma cacao* F3H (SEQ ID NO: 14); and *Petunia x hybrida* F3H (SEQ ID NO: 16).

6. A genetically modified micro-organism according to any one of claims 1-5, wherein the polypeptide having flavonol synthase (FLS) activity comprises an amino acid sequence having at least 80% sequence identity to an amino acid sequence selected from among any one of *Arabidopsis thaliana* FLS (SEQ ID NO: 18); *Arabidopsis lyrata* FLS (SEQ ID NO: 20); *Capsella rubella* FLS (SEQ ID NO: 22); *Eutrema salsugineum* FLS (SEQ ID NO: 24); *Morus notabilis* FLS (SEQ ID NO: 26); *Citrus unshiu* FLS (SEQ ID NO: 28); *Theobroma cacao* FLS (SEQ ID NO: 30); *Vitis vinifera* FLS (SEQ ID NO: 32); and *Eucalyptus grandis* FLS (SEQ ID NO: 34).

7. A genetically modified micro-organism according to any one of claim 1-2, wherein the polypeptide having flavonoid 3'-monooxygenase (FMO) activity comprises an amino acid sequence having at least 80% sequence identity to an amino acid sequence selected from among any one of *Arabidopsis thaliana* FMO (SEQ ID NO: 36); Fragaria x ananassa FMO 1 and 2 (SEQ ID No:40 and SEQ ID No: 42); *Petunia hybrida* FMO (SEQ ID NO: 38); and *Malus x domestica* FMO (SEQ ID No. 44);

Eutrema salsugineum (SEQ ID NO: 46); *Capsella rub*ella (SEQ ID NO: 48); *Nicotiana tabacum* (SEQ ID NO: 50); and *Vitis vinifera* (SEQ ID NO: 52).

8. A genetically modified micro-organism according to any one of claims 1-7, wherein the polypeptide having cytochrome P450 reductase (CPR) activity comprises an amino acid sequence having at least 80% sequence identity to an amino acid sequence selected from among any one of *Catharanthus roseus* CPR (SEQ ID No: 54); *Arabidopsis thaliana* CPR (1) (SEQ ID NO: 56) or CPR (2) (SEQ ID NO: 58); *Petunia x hybrid* CPR (1) (SEQ ID NO: 60) and CPR (2) (SEQ ID NO: 62); *Vitis vinifera* CPR (SEQ ID NO: 64); and *Capsella rubella* CPR (SEQ ID NO: 66).

9. A genetically modified micro-organism according to any one of claims 2-8, wherein the polypeptide having tyrosine ammonia-lyase (TAL) comprises an amino acid sequence having at least 80% sequence identity to an amino acid sequence selected from among any one of *Rhodobacter sphaeroides* TAL (SEQ ID NO: 68); *Flavobacterium johnsoniae* TAL (SEQ ID NO: 70); *Saccharothrix espanaensis* TAL (SEQ ID NO: 72); *Herpetosiphon aurantiacus* TAL (SEQ ID NO: 74) and (SEQ ID NO: 76); *Rhodotorula mucilaginosa / Rhodotorula rubra* TAL (SEQ ID NO: 78) and *Streptomyces sp. Tu 4128* TAL (SEQ ID NO: 80).

10. A genetically modified micro-organism according to any one of claims 2-9, wherein the polypeptide having 4-coumaroyl-CoA lyase (4CL) activity comprises an amino acid sequence having at least 80% sequence identity to an amino acid sequence selected from among any one of *Petroselinum crispum* 4CL (SEQ ID NO: 82); *Lithospermum erythrorhizon* (SEQ ID NO: 84); *Streptomyces coelicolor* (SEQ ID NO: 96); *Paulownia fortunei* (SEQ ID NO: 88); *Fraxinus mandshurica* (SEQ ID NO: 90); *Nicotiana tabacum* (SEQ ID NO: 92); *Streptomyces violaceoruber* (SEQ ID NO: 94); Streptomyces coelicoflavus ZG0656 (SEQ ID NO: 96); and *Streptomyces violaceorubidus* (SEQ ID NO: 98).

11. A genetically modified micro-organism according to any one of claims 1-10, wherein the polypeptide having chalcone synthase (CHS) activity comprises an amino acid sequence having at least 80% sequence identity to an amino acid sequence selected from among any one of of *Arabidopsis thaliana* CHS (SEQ ID No: 102); *Petunia x hybrid* CHS (SEQ ID No: 100); *Fragaria x ananassa* CHS (SEQ ID No: 104); *Malus domestica* CHS (SEQ ID No: 106); *Vitis vinifer*a CHS (SEQ ID No: 108); *Hypericum androsaemum* CHS (SEQ ID No: 121); and *Nicotiana alata* CHS (SEQ ID No: 112).

12. A genetically modified micro-organism according to any one of claims 1-11, wherein the polypeptide having chalcone reductase (CHR) activity comprises an amino acid sequence having at least 80% sequence identity to an amino acid sequence selected from among any one of *Medicago sativa* CHR (SEQ ID No: 114); *Arabidopsis thaliana* CHR (SEQ ID No: 116); *Fragaria x ananassa* CHR (SEQ ID No: 118) and (SEQ ID No: 120).; *Malus domestica* CHR (SEQ ID No: 122); *Vitis vinifera* CHR (SEQ ID No: 126); *Astragalus mongholicus* CHR (SEQ ID No: 128); *Malus domestica* CHR (SEQ ID No: 124 and *Sesbania rostrata* CHR (SEQ ID No: 130).

13. A genetically modified micro-organism according to any one of claims 1-12, wherein the polypeptide having chalcone isomerase (CHI) activity comprises an amino acid sequence having at least 80% sequence identity to an amino acid sequence selected from among any one of *Medicago sativa* CHI (SEQ ID No: 132) and (SEQ ID No: 134).; *Lotus japonicus* CHI (SEQ ID No: 136); *Medicago truncatula* CHI (SEQ ID No: 138); *Glycine max* CHI (SEQ ID No: 140); *Glycyrrhiza uralensis* CHI (SEQ ID No: 142); *Pueraria montana* var. *lobata* CHI (SEQ ID No: 144); *Phaseolus vulgaris* CHI (SEQ ID No: 146); *Astragalus mongholicus* CHI (SEQ ID No: 148).

14. A genetically modified micro-organism according to any one of claims 2-4, wherein the polypeptide having cinnamate 4-hydroxylase activity comprises an amino acid sequence having at least 80% sequence identity to the amino acid sequence of *Arabidopsis thaliana* C4H (SEQ ID No: 152); and the polypeptide having NADPH:cytochrome P450 reductase activity comprises an amino acid sequence having at least 80% sequence identity to the amino acid sequence of *A. thaliana* CPR (SEQ ID No: 58) and *Saccharomyces cerevisiae* CYP5 (SEQ ID No. 154).

15. A genetically modified micro-organism according to any one of claims 1 – 14, wherein said micro-organism is selected from among a bacterium, a yeast, and a filamentous fungus.

16. A method for the production of fisetin, comprising the steps of:

    a. introducing a microorganism according to any one claim 1, 2, 5-14, into a growth medium to produce a culture;

    b. providing a substrate for the production of fisetin;

    c. recovering fisetin produced by said culture, and optionally

    d. purifying the recovered fisetin.

17. A method for the production of garbanzol and/or resokaempferol, comprising the steps of:

    a. introducing a microorganism according to claim 3 or 4 into a growth medium to produce a culture;

    b. providing a substrate for the production of garbanzol and/or resokaempferol;

    c. recovering garbanzol and/or resokaempferol produced by said culture,

    d. and optionally purifying the recovered garbanzol and/or resokaempferol.

18. Use of a genetically modified micro-organism according to any one of claims 1, 2, 5-15 for production of fisetin.

19. Use of a genetically modified micro-organism according to claim 4 or 5 for production of garbanzol and/or resokaempferol.

Figure 1

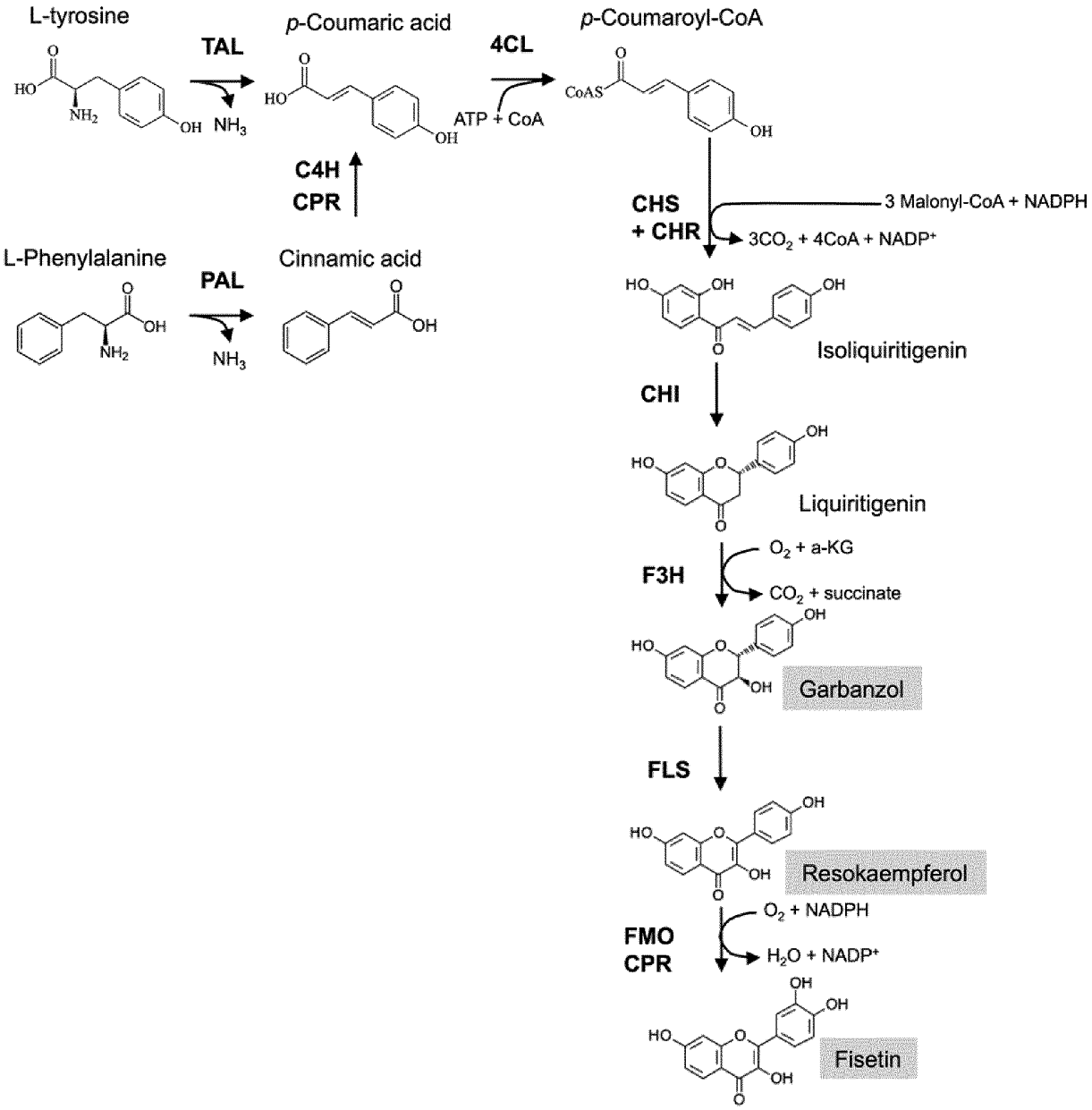

L-tyrosine
TAL
NH3
p-Coumaric acid
4CL
ATP + CoA
p-Coumaroyl-CoA
C4H
CPR
L-Phenylalanine
PAL
NH3
Cinnamic acid
CHS
+ CHR
3 Malonyl-CoA + NADPH
3CO2 + 4CoA + NADP+
Isoliquiritigenin
CHI
Liquiritigenin
F3H
O2 + a-KG
CO2 + succinate
Garbanzol
FLS
Resokaempferol
FMO
CPR
O2 + NADPH
H2O + NADP+
Fisetin

Figure 2

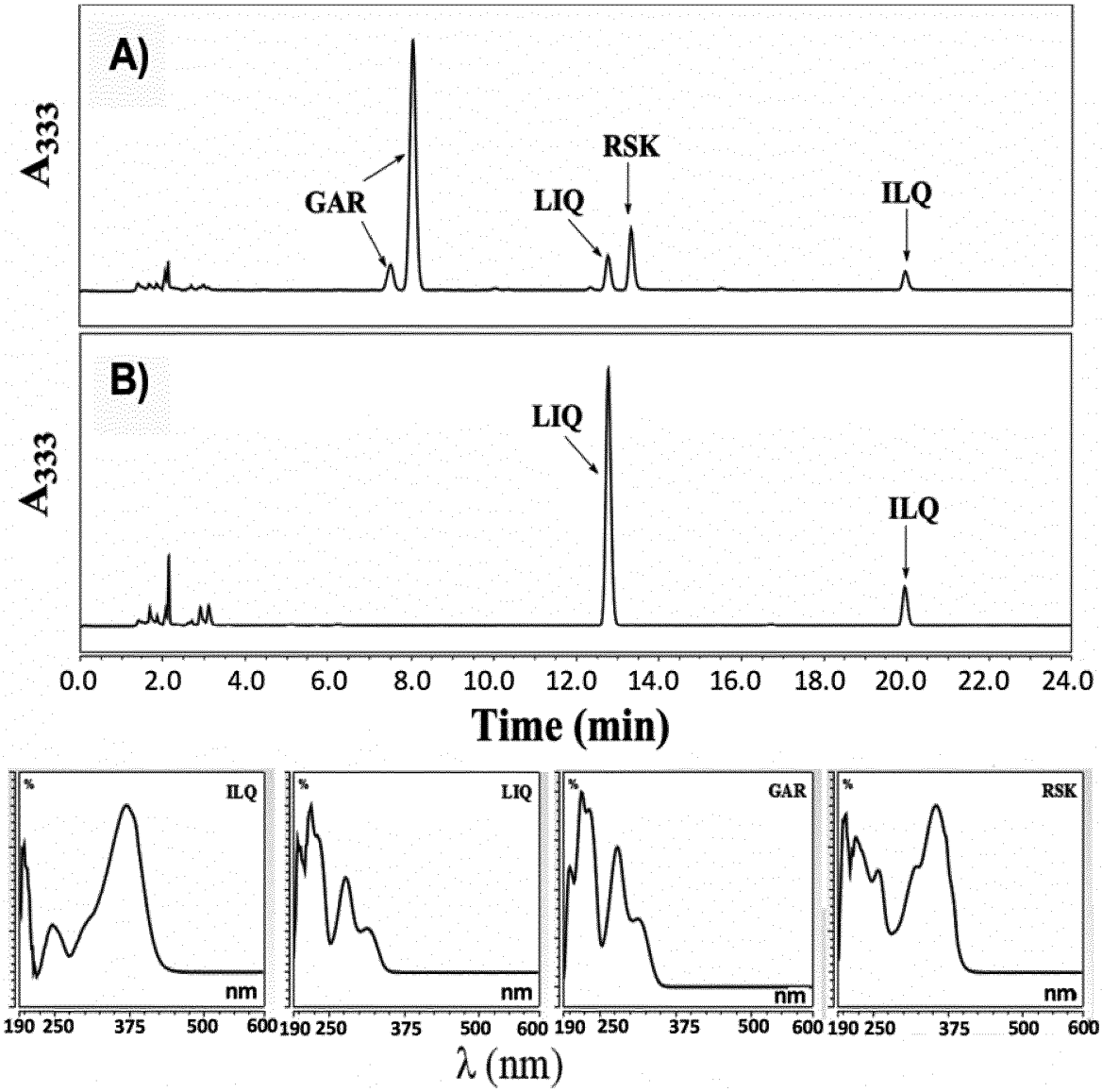

A)
A333
GAR
LIQ
RSK
ILQ
B)
A333
LIQ
ILQ
0.0 2.0 4.0 6.0 8.0 10.0 12.0 14.0 16.0 18.0 20.0 22.0 24.0
Time (min)
ILQ
LIQ
GAR
RSK
%
nm
190 250 375 500 600
λ (nm)

Figure 3

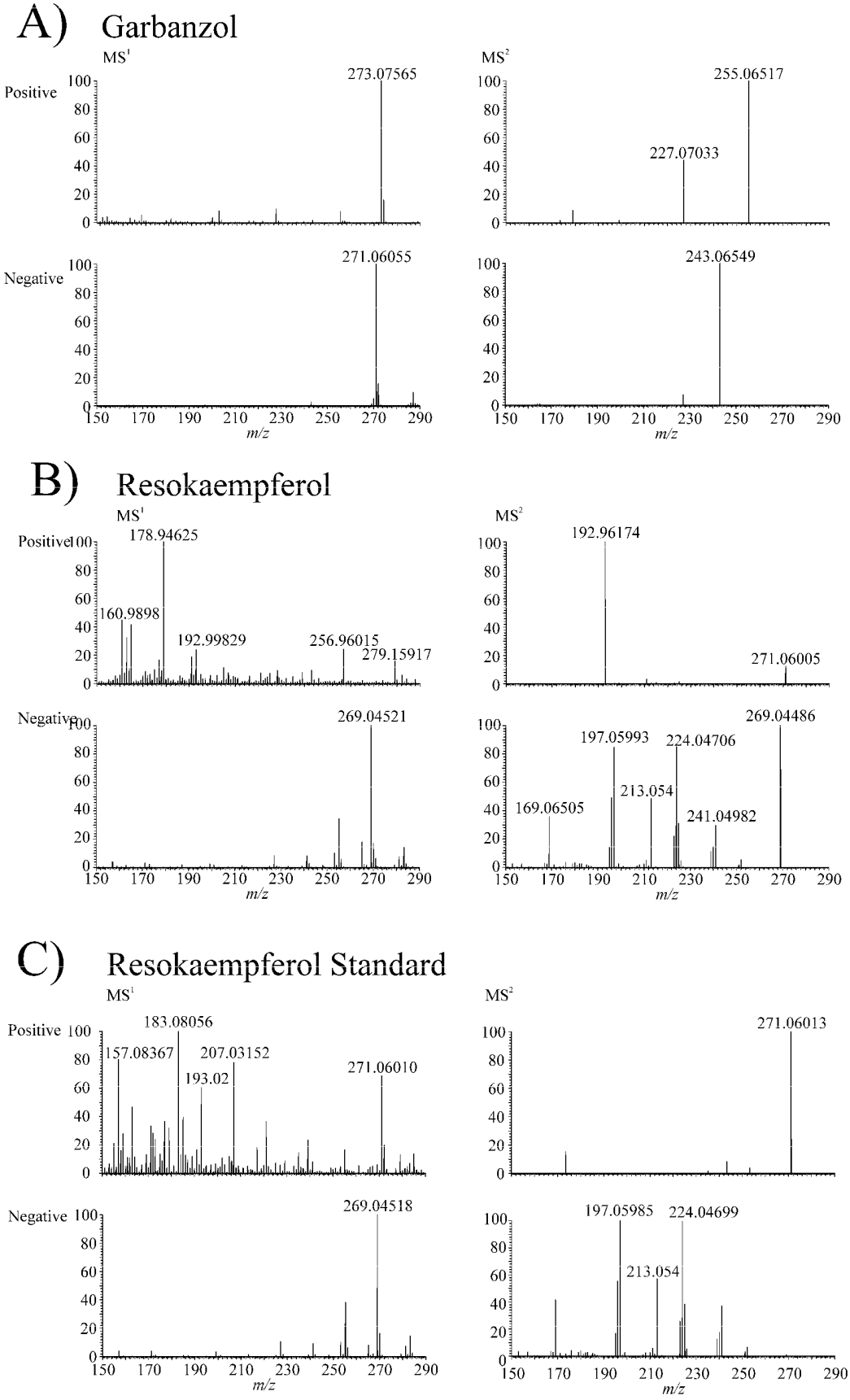
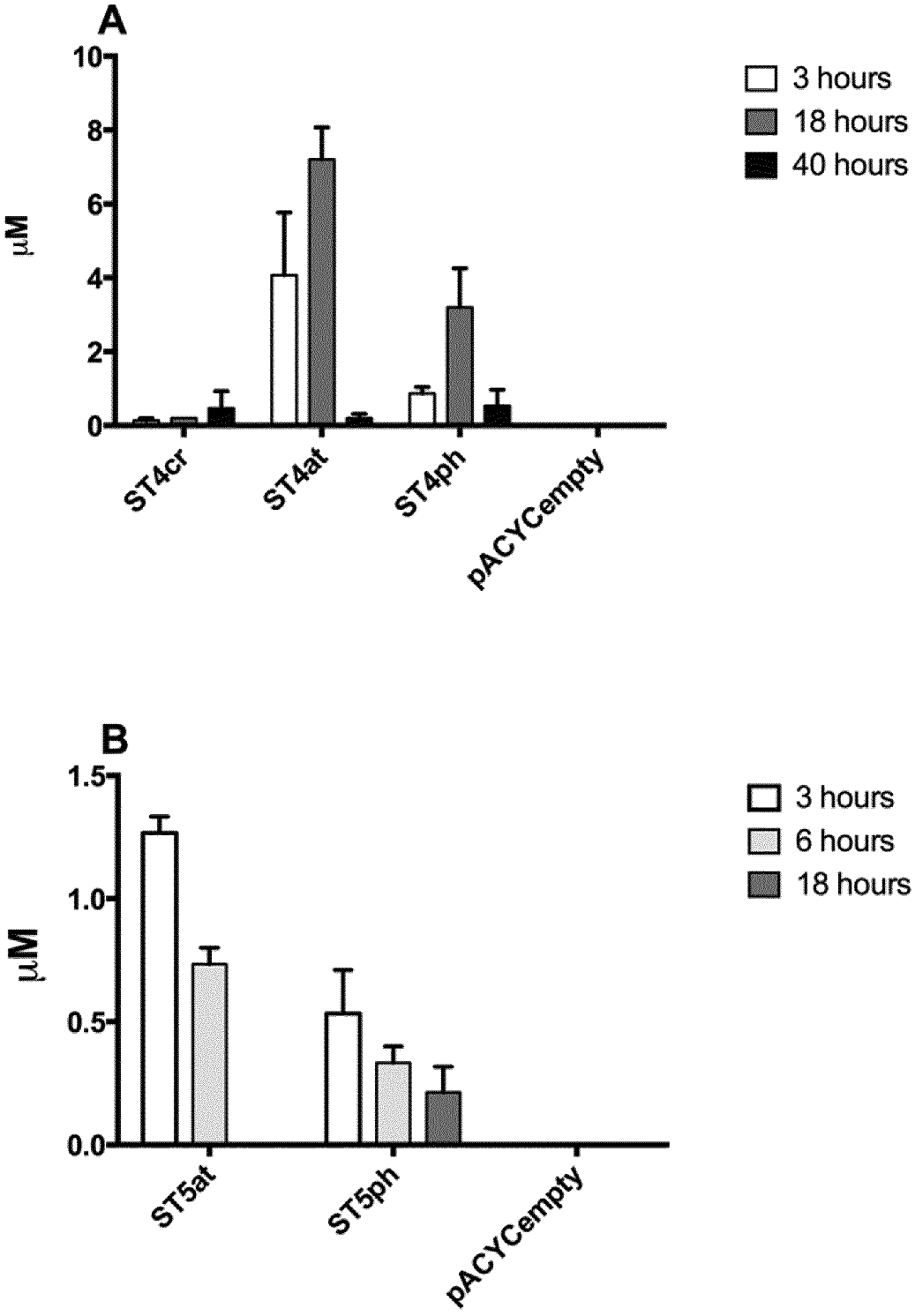

A) Garbanzol
MS1
MS2
Positive
273.07565
255.06517
227.07033
Negative
271.06055
243.06549
B) Resokaempferol
Positive
178.94625
160.9898
192.99829
256.96015
279.15917
192.96174
271.06005
Negative
269.04521
269.04486
197.05993
224.04706
213.054
169.06505
241.04982
C) Resokaempferol Standard
Positive
183.08056
157.08367
207.03152
193.02
271.06010
271.06013
Negative
269.04518
197.05985
224.04699
213.054
m/z

Figure 4


A
10
8
6
4
2
0
µM
3 hours
18 hours
40 hours
ST4cr
ST4at
ST4ph
pACYCempty
B
1.5
1.0
0.5
0.0
µM
3 hours
6 hours
18 hours
ST5at
ST5ph
pACYCempty

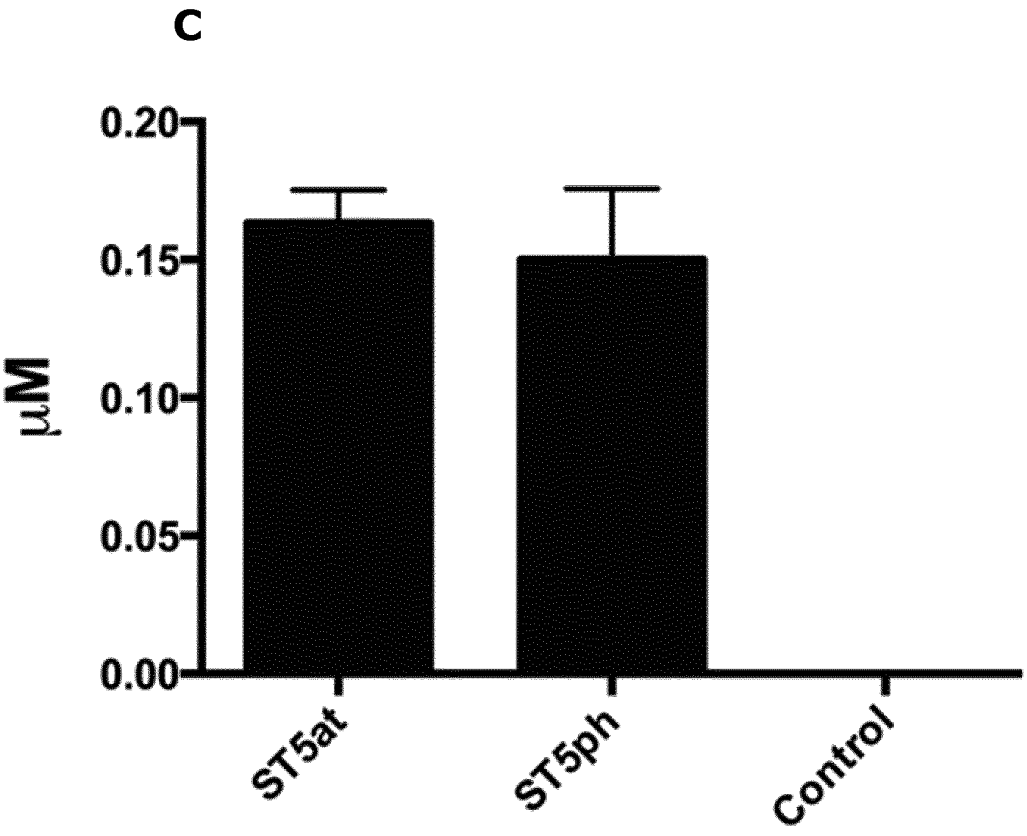

C
0.20
0.15
0.10
0.05
0.00
µM
ST5at
ST5ph
Control

Figure 5

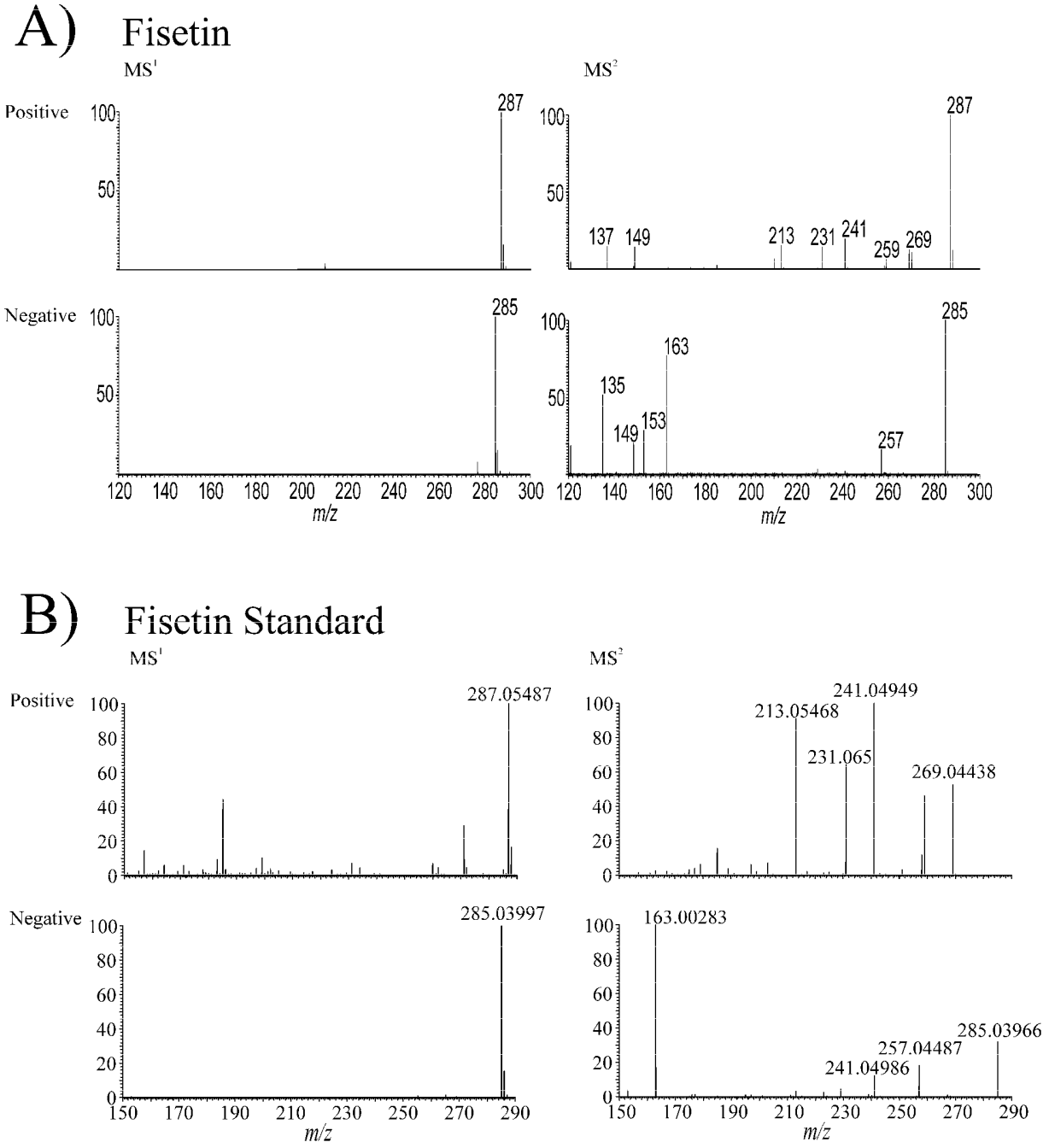

A) Fisetin
MS1
MS2
Positive
Negative
287
285
137 149 213 231 241 259 269
135 149 153 163 257
m/z
B) Fisetin Standard
MS1
MS2
Positive
Negative
287.05487
285.03997
241.04949
213.05468
231.065
269.04438
163.00283
285.03966
257.04487
241.04986
m/z

Figure 6

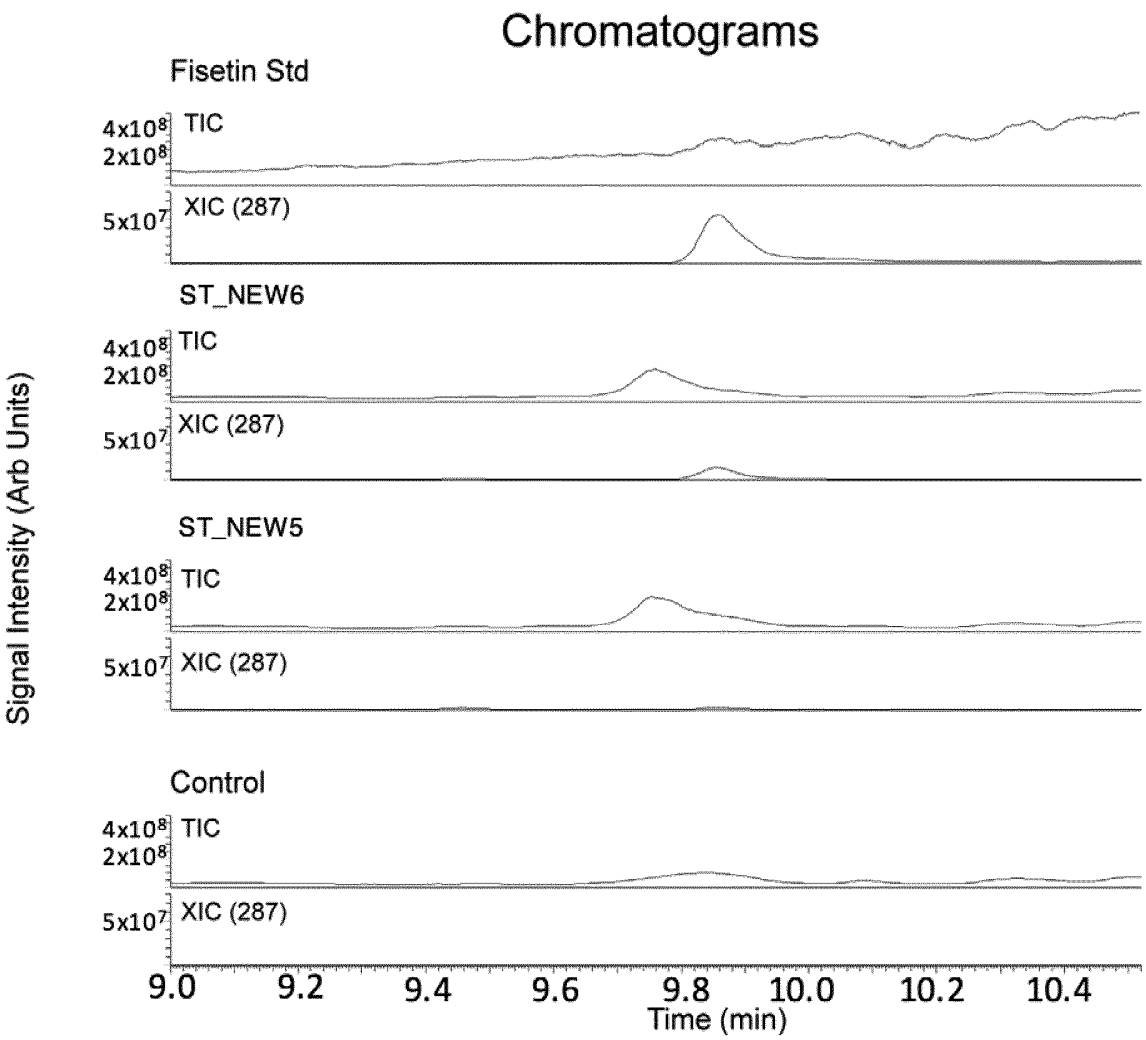
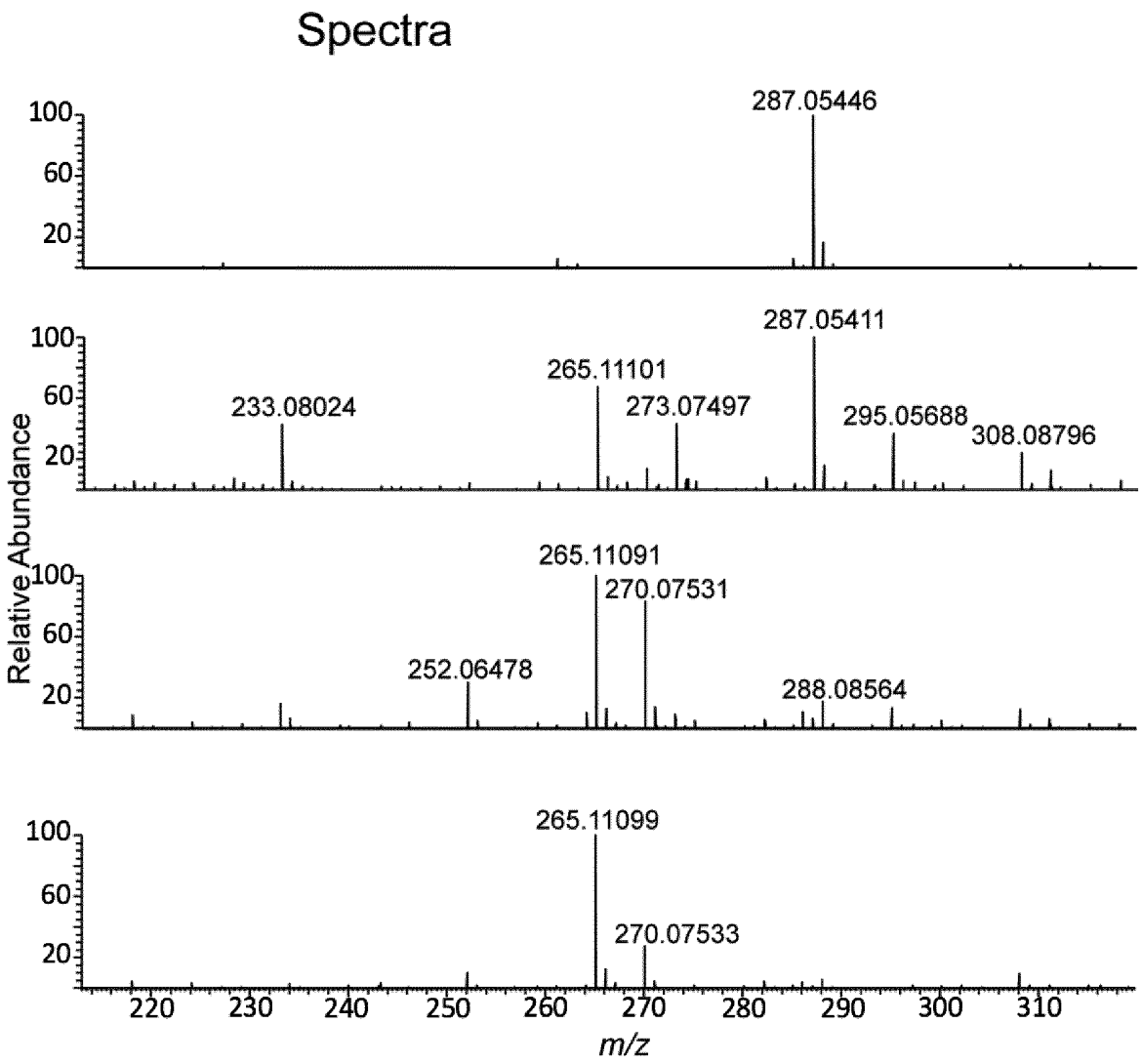

Chromatograms
Fisetin Std
TIC
XIC (287)
ST_NEW6
TIC
XIC (287)
ST_NEW5
TIC
XIC (287)
Control
TIC
XIC (287)
4x10^8
2x10^8
5x10^7
Signal Intensity (Arb Units)
9.0
9.2
9.4
9.6
9.8
10.0
10.2
10.4
Time (min)

Figure 6 continued



INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP2015/075953

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 sequence listing:

   a. [X] forming part of the international application as filed:

      [X] in the form of an Annex C/ST.25 text file.

      [ ] on paper or in the form of an image file.

   b. [ ] furnished together with the international application under PCT Rule 13*ter*.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.

   c. [ ] furnished subsequent to the international filing date for the purposes of international search only:

      [ ] in the form of an Annex C/ST.25 text file (Rule 13*ter*.1(a)).

      [ ] on paper or in the form of an image file (Rule 13*ter*.1(b) and Administrative Instructions, Section 713).

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 forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2015/075953

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12P17/06 C07D311/30
ADD.

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)
C12P C07D

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, WPI Data, Sequence Search, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	IKUO MIYAHISA ET AL: "Combinatorial biosynthesis of flavones and flavonols in Escherichia coli", APPLIED MICROBIOLOGY AND BIOTECHNOLOGY, SPRINGER, BERLIN, DE, vol. 71, no. 1, 18 August 2005 (2005-08-18), pages 53-58, XP019421874, ISSN: 1432-0614 cited in the application	3-6,8-19
A	abstract figure 1 page 54, left-hand column, paragraph 1	1,2,7

-/--

[X] Further documents are listed in the continuation of Box C.

[X] 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	Date of mailing of the international search report
3 March 2016	10/03/2016

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Niebuhr-Ebel, K

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Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WATTS K T ET AL: "Exploring recombinant flavonoid biosynthesis in metabolically engineered Escherichia coli", CHEMBIOCHEM - A EUROPEAN JOURNAL OF CHEMICAL BIOLOGY, WILEY VCH, WEINHEIM, DE, vol. 5, no. 4, 2 April 2004 (2004-04-02), pages 500-507, XP002347112, ISSN: 1439-4227, DOI: 10.1002/CBIC.200300783 the whole document	1-19
A	MASAFUMI KANEKO ET AL: "Heterologous production of flavanones in Escherichia coli : potential for combinatorial biosynthesis of flavonoids in bacteria", JOURNAL OF INDUSTRIAL MICROBIOLOGY & BIOTECHNOLOGY, vol. 30, no. 8, 1 August 2003 (2003-08-01) , pages 456-461, XP055003953, ISSN: 1367-5435, DOI: 10.1007/s10295-003-0061-1 the whole document	1-19
A	MARÍA L. FALCONE FERREYRA ET AL: "Flavonoids: biosynthesis, biological functions, and biotechnological applications", FRONTIERS IN PLANT SCIENCE, vol. 3, no. 222, 1 January 2012 (2012-01-01), XP055183106, ISSN: 1664-462X, DOI: 10.3389/fpls.2012.00222 the whole document	1-19
A	WO 01/51482 A1 (BIOREX HEALTH LTD [AU]; WALLACE ROBERT GERARD [AU]; BURONG WILLFRITS G) 19 July 2001 (2001-07-19) page 3, line 10 - page 6, line 4 claim 21	1-19
T	STEEN G. STAHLHUT ET AL: "Assembly of a novel biosynthetic pathway for production of the plant flavonoid fisetin in Escherichia coli", METABOLIC ENGINEERING, vol. 31, 1 September 2015 (2015-09-01), pages 84-93, XP055242058, US ISSN: 1096-7176, DOI: 10.1016/j.ymben.2015.07.002 the whole document	

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2015/075953

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 0151482 A1	19-07-2001	AT 307126 T	15-11-2005
		BR 0107486 A	08-10-2002
		CA 2396734 A1	19-07-2001
		CN 1395573 A	05-02-2003
		DE 60114151 D1	24-11-2005
		DE 60114151 T2	20-07-2006
		DK 1254131 T3	06-03-2006
		EP 1254131 A1	06-11-2002
		ES 2251458 T3	01-05-2006
		JP 2004500374 A	08-01-2004
		NZ 519931 A	31-01-2003
		WO 0151482 A1	19-07-2001