

METHODS FOR TUNING CAROTENOID PRODUCTION LEVELS AND COMPOSITIONS IN *RHODOSPORIDIUM* AND *RHODOTORULA* GENERA

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] The present application is related to and claims priority to U.S. Provisional Patent Application Serial No. 62/091,913 filed on 15 December 2014. This application is incorporated herein by reference in its entirety.

SEQUENCE SUBMISSION

[0002] The present application is being filed along with a Sequence Listing in electronic format. The Sequence Listing is entitled 2577244PCTSequenceListing.txt, created on 03 November 2015 and is 184 kb in size. The information in the electronic format of the Sequence Listing is incorporated herein by reference in their entirety.

BACKGROUND OF THE INVENTION

[0003] The present invention relates to the field of fungal biotechnology, more particularly to genetic engineering methods for the production of carotenoids in fungal hosts selected from *Rhodospordium* and *Rhodotorula* genera.

[0004] The publications and other materials used herein to illuminate the background of the invention, and in particular, cases to provide additional details respecting the practice, are incorporated by reference, and for convenience are referenced in the following text by author and date and are listed alphabetically by author in the appended bibliography.

[0005] It is well documented that carotenoid production is initiated with the biosynthesis of geranylgeranyl diphosphate (GGPP) catalyzed by GGPP synthase for the condensation of C₁₅ farnesyl diphosphate (FPP) and C₅ isopentenyl diphosphate (IPP). Subsequently, two molecules of GGPP are further condensed to form the colorless precursor phytoene, which is catalyzed by phytoene synthase. In fungi and eubacteria, phytoene desaturase catalyzes all 4 steps of desaturation of phytoene to yield the red colored lycopene while this is done by separate phytoene desaturase and γ -carotene or turase and desaturase in plant, algae and cyanabacteria. Lycopene is cyclized by carotene cyclase to form mono-cyclic γ -carotene and teneene cy, and dicyclic α -carotene and carotene a [1, 2]. Further upstream of the biosynthetic pathway, FPP is produced by farnesyl diphosphate synthase (FPS) catalyzed condensation of IPP and C₁₀ geranyl diphosphate (GPP), the latter produced by GPP synthase catalyzed condensation of IPP and

dimethylallyl diphosphate (DMAPP), the product of IPP isomerase (IPI). IPP and DMAPP can be synthesized via either the mevalonate pathway (MVP) or 2-C-methyl-D-erythritol 4-phosphate/1-deoxy-D-xylulose 5-phosphate pathway (MEP/DOXP) [3, 4].

[0006] Carotenoids are 40 carbon (C₄₀) tetraterpenoids [5]. The unoxygenated carotenoids, such as γ -carotene, β -carotene and lycopene are known as carotenes. Further enzymatic modifications of carotenes produce molecules containing oxygen, such as lutein, retinol (vitamin A), zeaxanthin and astaxanthin [6, 7]. Biosynthesis of carotenoids occur in all photosynthetic organisms [8] and many non-photosynthetic microorganisms, such as bacteria and fungi [1, 5, 9, 10], and some insects [11].

[0007] Carotenoids play important role in human and animal health and development [12-15]. For example, a higher dietary intake of carotenoids was associated with a lower risk for age-related macular degeneration (AMD) [13]; vitamin A deficiency is associated with abnormal growth of the skeleton and teeth and infertility in rat [14]; retinal (retinaldehyde) is essential for vision while retinoic acid is essential for skin health, teeth remineralization and bone growth [16]; intakes of lycopene is related to lower risk prostate cancer [17]. Carotenoids are natural colorant with many colors available [18-20]. Carotenoids are precursors for the production of valuable aromatic compounds [21]. β -carotene can be cleaved by P450 cytochrome oxidase to make retinal (retinaldehyde) [16], which is essential for vision and when converted to retinoic acid, it is essential for skin health, teeth remineralization and bone growth. Therefore, carotenoids are valuable food and feed additives, nutraceuticals and cosmoceutical.

[0008] Retinol, retinal and retinoic acid are known as retinoids, which are derived from breakdown of skin health, teeth remineralization and bone growth. Retinol and retinal is interconvertible and catalyzed by alcohol dehydrogenase and short-chain dehydrogenase/reductases whereas aldehyde dehydrogenase and cytochrome P450 enzyme families catalyze the irreversible oxidation of retinal to retinoic acid. The identification of enzymes catalyzing retinol oxidation *in vivo* has been controversial, in part due to the difficulty by the reversible nature of this reaction [22].

[0009] *Rhodospiridium* and *Rhodotorula* are two fungal genera belonging to the *Pucciniomycotina* subphyla. They can be cultured in single-cell form in very high cell density in fermentors at a fast growth rate and accumulate high levels of triacylglyceride [23-26]. *Rhodospiridium* and *Rhodotorula* are able to produce high levels of carotenoid [27-30], with beta-carotene, gamma-carotene, torularhodin and torulene being the major components [31]. Torularhodin and torulene are potential colorants and inducer of gene expression. Apart from the

identification of a putative *CAR2* homolog [32], there is no report on the carotenoid biosynthetic pathway in *Rhodospordium* and *Rhodotorula*. Any method that improves the productivity and product purity of carotenoids and their derivatives are of commercial value and significance.

SUMMARY OF THE INVENTION

[0010] The present invention relates to the field of fungal biotechnology, more particularly to genetic engineering methods for the production of carotenoids in fungal hosts selected from *Rhodospordium* and *Rhodotorula* genera.

[0011] Thus in one aspect, the present invention provides a method for tuning the production level and composition of carotenoids in a fungal host. In accordance with this aspect, the method comprises genetic manipulation of one or more of polynucleotides involved in carotenoid biosynthesis. In one embodiment, the carotenoids are lycopene, beta-carotene, gamma-carotene, torulene, torularhodin or derivatives thereof. In some embodiments, a derivative is a hydroxylated derivative, a glycosylated derivative or an oxidated derivative. In another embodiment, the fungal host is *Rhodospordium* or *Rhodotorula*. In some embodiments, the method comprises genetically manipulating one or more polynucleotides involved in carotenoid biosynthesis in a fungal host and growing the fungal host to produce the carotenoids, whereby the production level or composition of the carotenoids is tuned.

[0012] In one embodiment, the genetic manipulation comprises the down-regulation of one or more polynucleotides selected from SEQ ID NOs:1, 3, 5, 7, 8, 9, 10, 11, 13, 14, 16, 17, 19 and 20, or a homolog sharing at least 75% nucleotide identity thereto in a fungal host. In another embodiment, the genetic manipulation comprises the down-regulation of one or more polynucleotides encoding polypeptides selected from SEQ ID NOs:2, 4, 6, 12, 15, 18 and 21 or a homolog sharing at least 75% identity thereto in a fungal host. In some embodiments, the down-regulation is compared to a fungal host without the genetic manipulation. In other embodiments, the one or more polynucleotides are down-regulated by RNAi, artificial transcriptional repressor or a weak promoter.

[0013] In one embodiment, the genetic manipulation is the total inactivation of enzyme function in a fungal host. In some embodiments the inactivation is achieved by deletion of all or a part of one or more polynucleotides selected from SEQ ID NOs:10, 11, 16, 17, 19 and 20 or a homolog sharing at least 75% nucleotide identity thereto. In other embodiments the inactivation is achieved by deletion of all or a part of one or more polynucleotides encoding polypeptides selected from SEQ ID NOs:12, 18 and 21 or a homolog sharing at least 75% nucleotide identity

thereto. In one embodiment, the deletion is performed by using homologous recombination technique. In another embodiment, the deletion is aided by using an artificial nuclease. In a further embodiment, the artificial nuclease is a Zinc finger Nuclease (ZFN) or Cas9-gRNA complex.

[0014] In one embodiment, the genetic manipulation involves over-expression of one or more polynucleotides selected from SEQ ID NOs:1, 3, 5, 7, 8, 9, 10, 11, 13, 14, 16, 17, 19 and 20, or a homolog sharing at least 75% nucleotide identity thereto. In another embodiment, the genetic manipulation involves over-expression of one or more polynucleotides encoding polypeptides selected from SEQ ID NOs:2, 4, 6, 12, 15, 18 and 21; or a homolog sharing at least 75% identity thereto. In some embodiments, the over-expression is mediated by introduction of a synthetic gene cassette into a fungal host cell. In other embodiment, the cassette comprises a heterologous promoter operably linked to the polynucleotide, optionally operably linked to a transcriptional terminator. In some embodiments, the over-expression is compared to a fungal host without the genetic manipulation.

[0015] In some embodiments, the genetic manipulation involves a combination of the previously described genetic manipulations. In one embodiment, the genetic manipulation comprises the down-regulation of one or more of the polynucleotides and over-expression of one or more different polynucleotides. In another embodiment, the genetic manipulation comprises the total inactivation of enzyme function of one or more polypeptides encoded by one or more polynucleotides and the over-expression of one or more of different polynucleotides. In a further embodiment, the genetic manipulation comprises the total inactivation of enzyme function of one or more polypeptides encoded by one or more polynucleotides and the down-regulation of one or more different polynucleotides. In an additional embodiment, the genetic manipulation comprises the total inactivation of enzyme function of one or more polypeptides encoded by one or more polynucleotides, the over-expression of one or more of different polynucleotides and the down-regulation of one or more different polynucleotides.

[0016] In some embodiments, the polynucleotides described herein that have been stably incorporated in the fungal genome are operatively linked to a promoter which permits efficient expression in species of the *Rhodospordium* genera and the *Rhodotorula* genera. The promoters for each incorporated polynucleotide may be the same or different. In some embodiments, the promoters are promoters found in species of the *Rhodospordium* genera and the *Rhodotorula* genera. Examples of suitable promoters include, but are not limited to, promoters of the following genes encoding the following proteins: glyceraldehyde 3-phosphate dehydrogenase

(GPD), acyl-CoA carrier protein (ACP), fatty acid desaturase, translation elongation factor (TEF), pyruvate decarboxylase (PDC), enolase (2-phosphoglycerate dehydratase) (ENO), peptidylprolyl isomerase (PPI), acetyl-CoA carboxylase (ACC) or transaldolase. In other embodiments, the genes described herein also include a mRNA transcriptional terminator that may be one found in any eukaryotic species and their DNA viruses.

[0017] In another embodiment, the present invention provides a method for producing carotenoids which comprises growing a fungal host cell described herein under conditions suitable to produce carotenoids. Any medium with at least 5% carbon source can be used. In some embodiments, the carbon source is glucose, mannose, glycerol, sucrose, xylose or combinations thereof. In one embodiment, the medium is Medium MinCAR containing 30-100 g glucose, 1.5 g yeast extract, 0.5 g (NH₄)₂SO₄, 2.05 g K₂HPO₄, 1.45 g KH₂PO₄, 0.6 g MgSO₄, 0.3 g NaCl, 10 mg CaCl₂, 1 mg FeSO₄, 0.5 mg ZnSO₄, 0.5 mg CuSO₄, 0.5 mg H₃BO₄, 0.5 mg MnSO₄, 0.5 mg NaMoO₄ (per liter). The medium is preferably adjusted to pH 5-7. In some embodiments the cell culturing is preferably performed at 25°-35°C. In other embodiments, the culturing is preferably performed in a condition with lighting.

BRIEF DESCRIPTION OF THE FIGURES

[0018] Fig. 1 shows the organization of pRH201. LB: left border of T-DNA; RB: right border of T-DNA; *P_{gpd}*: 595 bp promoter of *Um_{gpd1}*; *P_{GPD1}*: 795 bp promoter of *Rt_{GPD1}*; *hpt-3*: codon-optimized hygromycin resistance gene based on the codon usage bias in *R. toruloides*; Tnos: terminator of *A. tumefaciens* nopaline synthase gene. Unique restriction enzymes cutting sites are shown in red. loxP-RE and loxP-LE and mutant recognition sites for Cre recombinase. Sp/Str are resistance gene for spectinomycin and streptomycin; eGFP-His6 is a *R. toruloides* codon-adapted gene encoding eGFP-histidine tag fusion protein; 35S: *cauliflower mosaic virus* 35S gene terminator.

[0019] Figs. 2A-2E show the identification of RCM mutants and characterization of *CAR1* gene. Fig. 2A: Colony color phenotypes of RCM mutants. All strains were streaked on PDA plate and incubated at 28°C for 2 days. Fig. 2B: Schematic diagram of *CAR1* and its deletion strategy. Fig. 2C: Southern blot analysis of candidate *CAR1* null mutant ($\Delta car1$). Homologous sequences used for deletion of *CAR1* were 1036 bp (*CAR1L*) and 830 bp (*CAR1R*) in length, ranging from -89 to +947 and +2098 to +2928 of the translational start codon. Digoxigenin labeled DNA fragment *CAR1R* was used as the probe for the detection *Hind*III total DNA. Fig. 2D: Colony colors of WT, null mutant ($\Delta car1$) and complementation strain ($\Delta car1C$) cultured

on PDA plate. Fig. 2E: Carotenoid profiles in *R. toruloides* wild type strain, $\Delta car1$ and $\Delta car1C$. The content of four major carotenoid components were analyzed.

[0020] Figs. 3A-3D show the carotenoid biosynthetic gene cluster in *R. toruloides*. Fig. 3A: Genomic organization of carotenoid biosynthesis gene clusters in 5 carotenogenic fungi, *Blakeslea trispora*, *Fusarium fujikuroi*, *Phycomyces blakesleeanus* and *Sporobolomyces roseus*. Fig. 3B: Deletion of *CAR3*, *CCD1* and *CDS1*. Upper panel shows the deletion schemes and lower panel shows the Southern blot analysis of knockout mutants. The black bars indicate the probes used for Southern blot hybridization. Fig 3.C: Colony color phenotype of null mutants involved in carotenoid biosynthesis pathway in *R. toruloides*. Fig. 3D: Carotenoid profiles in *R. toruloides* wild type strain and null mutants of *CCD1* and *CDS1* genes.

[0021] Fig. 4 shows carotenoid profiles in *R. toruloides* wild type strain, Ald1 null mutant (*ald1*) and overexpression of Ald1OE.

[0022] Fig. 5 shows relative mRNA levels of carotenoid biosynthetic genes after switching to lighting. Expression level of each gene was done by qRT-PCR and normalized to Actin gene (*ACT1*).

[0023] Figs. 6A-6C show the characterization of *Roc1*. Fig 6A: Schematic structure of *ROCI* and gene deletion strategy. Fig. 6B: Phylogenetic tree analysis of negative regulators of fungal carotenoid biosynthesis. NCBI GenBank accession number was followed by the gene name. Fig. 6C: Comparison of RING-finger domains and LON domains. The consensus sequences are indicated in the bottom line of each. GenBank accession numbers (with sequences in Fig. 6C set forth in the indicated sequences): *B. trispora* *crgA*: CAE51310.1 (SEQ ID NO:26); *M. circinelloides* *crgA*: CAB61339.2 (SEQ ID NO:28); *A. fumigatus* *crgA*: XP_755380.1 (SEQ ID NO:25); *F. fujikuroi* *carS*: CCP50075.1 (SEQ ID NO:27); *P. blakesleeanus* *carS*: ADU04395.1 (SEQ ID NO:29); *U. maydis*: EAK85777.1 (SEQ ID NO:31); *R. toruloides*: (SEQ ID NO:30).

[0024] Figs. 7A-7E show deletion of *ROCI*. Fig. 7A: Colony color differences between ectopic and homologous recombination of knockout *ROCI*. Fig. 7B: Southern blot verification of gene deletion mutants. Digoxigenin-labeled DAN Molecular Weight Marker VII (Roche Diagnosis, USA) was used as the marker. Fig. 7C: Cell morphology of *roc1* null mutant and wild type. Bar represents 10 μ m. Fig. 7D: mRNA transcripts of carotenoid genes in *roc1* mutant and wild type strain under illumination condition. Fig. 7E: Comparison of carotenoid production in wild type, knockout mutant strain ($\Delta roc1$) and its complementation strain ($\Delta roc1C$).

DETAILED DESCRIPTION OF THE INVENTION

[0025] The present invention relates to the field of fungal biotechnology, more particularly to genetic engineering methods for the production of carotenoids in fungal hosts selected from *Rhodospordium* and *Rhodotorula* genera.

[0026] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as is commonly understood by one of skill in the art to which the invention belongs.

[0027] The term “about” or “approximately” means within a statistically meaningful range of a value. Such a range can be within an order of magnitude, preferably within 50%, more preferably within 20%, more preferably still within 10%, and even more preferably within 5% of a given value or range. The allowable variation encompassed by the term “about” or “approximately” depends on the particular system under study, and can be readily appreciated by one of ordinary skill in the art.

[0028] As used herein, “allele” refers to any of one or more alternative forms of a gene locus, all of which alleles relate to a trait or characteristic. In a diploid cell or organism, the two alleles of a given gene occupy corresponding loci on a pair of homologous chromosomes.

[0029] The term "down regulation" refers to diminishment in the level of expression of a polynucleotide, such as a gene, compared to a control using any method known in the art, such as RNAi, an artificial transcriptional repressor to specifically target the gene of interest, such as ZFN and Cas9 that is fused to a transcriptional repressor domain and bind to specific DNA sequence in a gene's promoter or coding sequence to achieve the down-regulation; or use of a weaker promoter to drive the expression of the gene of interest. The term "down regulated" is used herein to indicate that the target gene expression is lowered by 1-100%. For example, the expression may be reduced by about 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 99%.

[0030] As used herein, a "comparison window" or “window of comparison” refers to a conceptual segment of at least 6 contiguous positions, usually about 50 to about 100, more usually about 100 to about 150, in which a sequence is compared to a reference sequence of the same number of contiguous positions after the two sequences are optimally aligned. The comparison window may comprise additions or deletions (i.e. gaps) of about 20% or less as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. Those skilled in the art should refer to the detailed methods used for sequence alignment, such as in the Wisconsin Genetics Software Package Release 7.0 (Genetics Computer Group, 575 Science Drive Madison, Wisc., USA).

[0031] A "dsRNA" or "RNAi molecule," as used herein in the context of RNAi, refers to a compound, which is capable of down-regulating or reducing the expression of a gene or the activity of the product of such gene to an extent sufficient to achieve a desired biological or physiological effect. The term "dsRNA" or "RNAi molecule," as used herein, refers to one or more of a dsRNA, siRNA, miRNA, hpRNA, ihpRNA.

[0032] The term "expression" with respect to a gene sequence refers to transcription of the gene and, as appropriate, translation of the resulting mRNA transcript to a protein. Thus, as will be clear from the context, expression of a protein coding sequence results from transcription and translation of the coding sequence.

[0033] As used herein, "genotype" refers to the genetic constitution of a cell or organism.

[0034] The term "homolog" as used herein refers to a gene related to a second gene by descent from a common ancestral DNA sequence. The term, homolog, may apply to the relationship between genes separated by the event of speciation (ortholog) or to the relationship between genes separated by the event of genetic duplication (paralog). The term homolog is used generically to refer to all species.

[0035] "Operable linkage" or "operably linked" or "operatively linked" as used herein is understood as meaning, for example, the sequential arrangement of a promoter and the nucleic acid to be expressed and, if appropriate, further regulatory elements such as, for example, a terminator, in such a way that each of the regulatory elements can fulfill its function in the recombinant expression of the nucleic acid to make dsRNA. This does not necessarily require direct linkage in the chemical sense. Genetic control sequences such as, for example, enhancer sequences, can also exert their function on the target sequence from positions which are somewhat distant, or indeed from other DNA molecules (cis or trans localization). Preferred arrangements are those in which the nucleic acid sequence to be expressed recombinantly is positioned downstream of the sequence which acts as promoter, so that the two sequences are covalently bonded with one another. Regulatory or control sequences may be positioned on the 5' side of the nucleotide sequence or on the 3' side of the nucleotide sequence as is well known in the art.

[0036] The term "over-expression" refers to increase in the level of expression of a polynucleotide, such as a gene, compared to a control using any method known in the art, such as an artificial transcriptional activator to specifically target the gene of interest, such as ZFN and Cas9 that is fused to a transcriptional activator domain and bind to specific DNA sequence in a gene's promoter; or use of a stronger promoter to drive the expression of the gene of interest.

The term "over-expression" is used herein to indicate that the target gene expression is increased by 25% or more compared to a control. For example, the expression may be increased by about 25%, 50%, 100%, 200%, 500%, 1000% and so on.

[0037] As used herein, "phenotype" refers to the detectable characteristics of a cell or organism, which characteristics are the manifestation of gene expression.

[0038] The terms "polynucleotide," "nucleic acid" and "nucleic acid molecule" are used interchangeably herein to refer to a polymer of nucleotides which may be a natural or synthetic linear and sequential array of nucleotides and/or nucleosides, including deoxyribonucleic acid, ribonucleic acid, and derivatives thereof. It includes chromosomal DNA, self-replicating plasmids, infectious polymers of DNA or RNA and DNA or RNA that performs a primarily structural role. Unless otherwise indicated, nucleic acids or polynucleotide are written left to right in 5' to 3' orientation, Nucleotides are referred to by their commonly accepted single-letter codes. Numeric ranges are inclusive of the numbers defining the range.

[0039] The terms "polypeptide," "peptide," and "protein" are used interchangeably herein to refer to a polymer of amino acid residues. The terms apply to amino acid polymers in which one or more amino acid residue is an artificial chemical analog of a corresponding naturally occurring amino acid, as well as to naturally occurring amino acid polymers. Amino acids may be referred to by their commonly known three-letter or one-letter symbols. Amino acid sequences are written left to right in amino to carboxy orientation, respectively. Numeric ranges are inclusive of the numbers defining the range.

[0040] As used herein, the term "sequence identity," "sequence similarity" or "homology" is used to describe sequence relationships between two or more nucleotide sequences. The percentage of "sequence identity" between two sequences is determined by comparing two optimally aligned sequences over a comparison window such as the full length of a referenced SEQ ID NO:, wherein the portion of the sequence in the comparison window may comprise additions or deletions (i.e., gaps) as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. The percentage is calculated by determining the number of positions at which the identical nucleic acid base or amino acid residue occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison, and multiplying the result by 100 to yield the percentage of sequence identity. A sequence that is identical at every position in comparison to a reference sequence is said to be identical to the reference sequence and vice-versa. A first nucleotide sequence when observed in the 5' to 3'

direction is said to be a "complement" of, or complementary to, a second or reference nucleotide sequence observed in the 3' to 5' direction if the first nucleotide sequence exhibits complete complementarity with the second or reference sequence. As used herein, nucleic acid sequence molecules are said to exhibit "complete complementarity" when every nucleotide of one of the sequences read 5' to 3' is complementary to every nucleotide of the other sequence when read 3' to 5'. A nucleotide sequence that is complementary to a reference nucleotide sequence will exhibit a sequence identical to the reverse complement sequence of the reference nucleotide sequence. These terms and descriptions are well defined in the art and are easily understood by those of ordinary skill in the art.

[0041] The term "total inactivation of enzyme function", as used herein, refers to the complete loss of enzyme function or activity for a given enzyme or a given polypeptide having enzyme function or activity compared to a control in which the enzyme has not been inactivated. The enzyme function can be totally inactivated by deleting all or part of a polynucleotide encoding the polypeptide having enzyme function. "Part of a polynucleotide", as used herein with respect to inactivation of enzyme function, refers to a portion of the polynucleotide, the deletion of which is sufficient to cause total inactivation of enzyme function. A part of the polynucleotide could be a single nucleotide or multiple nucleotides as long as the deletion results in premature termination of the polypeptide or a frameshift mutation, each of which would result in an inactive polypeptide. A part of the polynucleotide could be a part that encodes at least 10% but less than 90% of the polypeptide, also resulting in loss of enzyme function.

[0042] The term "tuning", as used in one embodiment herein, refers to either increasing or decreasing the yield of a carotenoid by at least 25%, by at least 30%, by at least 35%, by at least 40%, by at least 45%, or by about 50% or more in a genetically manipulated fungal host compared to a non-genetically manipulate control fungal host. The term "tuned", as used in one embodiment herein, refers to either increased or decreased yield of a carotenoid by at least 25%, by at least 30%, by at least 35%, by at least 40%, by at least 45%, or by about 50% or more in a genetically manipulated fungal host compared to a non-genetically manipulate control fungal host.

[0043] The term "tuning", as used in another embodiment herein, refers to a change in the composition of carotenoids in a genetically manipulated fungal host compared to a non-genetically manipulated control fungal host. The term "tuned", as used in another embodiment

herein, refers to a changed composition of carotenoids in a genetically manipulated fungal host compared to a non-genetically manipulated control fungal host.

[0044] Thus in one aspect, the present invention provides a method for tuning the production level and composition of carotenoids in a fungal host. In accordance with this aspect, the method comprises genetic manipulation of one or more of polynucleotides involved in carotenoid biosynthesis. In one embodiment, the carotenoids are lycopene, beta-carotene, gamma-carotene, torulene, torularhodin or derivatives thereof. In some embodiments, a derivative is a hydroxylated derivative, a glycosylated derivative or an oxidated derivative. In another embodiment, the fungal host is *Rhodospordium* or *Rhodotorula*. In some embodiments, the method comprises genetically manipulating one or more polynucleotides involved in carotenoid biosynthesis in a fungal host and growing the fungal host to produce the carotenoids, whereby the production level or composition of the carotenoids is tuned.

[0045] In one embodiment, the genetic manipulation comprises the down-regulation of one or more polynucleotides selected from SEQ ID NOs:1, 3, 5, 7, 8, 9, 10, 11, 13, 14, 16, 17, 19 and 20, or a homolog sharing at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98% or at least 99% nucleotide identity thereto in a fungal host. In another embodiment, the genetic manipulation comprises the down-regulation of one or more polynucleotides encoding polypeptides selected from SEQ ID NOs:2, 4, 6, 12, 15, 18 and 21, or a homolog sharing at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98% or at least 99% amino acid identity thereto in a fungal host. In some embodiments, the down-regulation is compared to a fungal host without the genetic manipulated. In other embodiments, the one or more polynucleotides are down-regulated by RNAi, artificial transcriptional repressor or a weak promoter.

[0046] Down-regulation of a polynucleotide of the present invention can be brought about by using well known techniques, including, but not limited to, RNAi techniques, such as dsRNA, miRNA, siRNA, smRNA, hpRNA or ihpRNA (collectively referred to as RNAi molecules), sense suppression (co-suppression), antisense, and the like. Such techniques are described in U.S. Patent Nos. 7,312,323 and references cited therein. For example, reduction might be accomplished, for example, with transformation of a fungal host cell to comprise a promoter and other 5' and/or 3' regulatory regions described herein linked to an antisense nucleotide sequence, hairpin, RNA interfering molecule, double stranded RNA, microRNA or other nucleic acid molecule, such that tissue-preferred expression of the molecule interferes with translation of the mRNA of the native DNA sequence or otherwise inhibits expression of the native DNA

sequence in plant cells. For further description of RNAi techniques or microRNA techniques, see, e.g., U.S. Patent Nos. 5,034,323; 6,326,527; 6,452,067; 6,573,099; 6,753,139; and 6,777,588. See also International Publication Nos. WO 97/01952, WO 98/36083, WO 98/53083, WO 99/32619 and WO 01/75164; and U.S. Patent Application Publication Nos. 2003/0175965, 2003/0175783, 2003/0180945, 2004/0214330, 2005/0244858, 2005/0277610, 2006/0130176, 2007/0265220, 2008/0313773, 2009/0094711, 2009/0215860, 2009/0308041, 2010/0058498 and 2011/0091975. RNAi molecules or microRNA molecules (referred to collectively herein as RNAi molecules) can be prepared by the skilled artisan using techniques well known in the art, including techniques for the selection and testing of RNAi molecules and microRNA molecules that are useful for down regulating a polynucleotide of the present invention.. See, for example, Wesley et al. (2001), Mysara et al. (2011) and Yan et al. (2012).

[0047] It has typically been found that dsRNA of 200-700 bp are particularly suited for inducing RNAi in plants. It has also been found that hairpin RNAs containing an intron, for example, a construct comprising an RNA encoding sequence in a sense direction operably linked to an intron operably linked to an RNA encoding sequence in an antisense direction or *vice versa* which is capable of forming an intron-hairpin RNA (ihpRNA), is suitable for inducing RNAi in plants. See, for example, Wang et al. (2000), Fuentes et al. (2006), Bonfim et al. (2007) Vanderschuren et al. (2007a, 2007b), Zrachya et al. (2007). For example, a nucleic acid construct can be prepared that includes a nucleic acid that is transcribed into an RNA that can anneal to itself, e.g., a double stranded RNA having a stem-loop structure. In addition, hairpin structures can be prepared as described by Guo et al. (2003).

[0048] For example, a nucleic acid construct can be prepared that includes a nucleic that is transcribed into an RNA that can anneal to itself, e.g., a double stranded RNA having a stem-loop structure. In some embodiments, one strand of the stem portion of a double stranded RNA comprises a sequence that is similar or identical to the sense coding sequence, or a fragment thereof, of a polynucleotide as described herein, and that is from about 10 nucleotides to about 1,800 nucleotides in length. The length of the sequence that is similar or identical to the sense coding sequence can be from 10 nucleotides to 1000 nucleotides, from 15 nucleotides to 600 nucleotides, from 20 nucleotides to 500 nucleotides, or from 25 nucleotides to 100 nucleotides, or any length within the 10 nucleotides to 2,500 nucleotides. The other strand of the stem portion of a double stranded RNA comprises a sequence that is similar or identical to the antisense strand, or a fragment thereof, of the coding sequence of the polypeptide of interest, and can have a length that is shorter, the same as, or longer than the corresponding length of the

sense sequence. In some cases, one strand of the stem portion of a double stranded RNA comprises a sequence that is similar or identical to the 3' or 5' untranslated region, or a fragment thereof, of the mRNA encoding a polypeptide described herein, and the other strand of the stem portion of the double stranded RNA comprises a sequence that is similar or identical to the sequence that is complementary to the 3' or 5' untranslated region, respectively, or a fragment thereof, of the mRNA encoding a polypeptide described herein. In other embodiments, one strand of the stem portion of a double stranded RNA comprises a sequence that is similar or identical to the sequence of an intron or a fragment thereof in the pre-mRNA transcribed from a polynucleotide described herein, and the other strand of the stem portion comprises a sequence that is similar or identical to the sequence that is complementary to the sequence of the intron or fragment thereof in the pre-mRNA.

[0049] The loop portion of a double stranded RNA can be from 3 nucleotides to 5,000 nucleotides, e.g., from 3 nucleotides to 2500 nucleotides, from 15 nucleotides to 1,000 nucleotides, from 20 nucleotides to 500 nucleotides, or from 25 nucleotides to 200 nucleotides, or any length within the 3 nucleotides to 5,000 nucleotides. The loop portion of the RNA can include an intron or a fragment thereof. A double stranded RNA can have zero, one, two, three, four, five, six, seven, eight, nine, ten, or more stem-loop structures.

[0050] In one embodiment, the genetic manipulation is the total inactivation of enzyme function in a fungal host. In some embodiments the inactivation is achieved by deletion of all or a part of one or more polynucleotides selected from SEQ ID NOs:10, 11, 16, 17, 19 and 20, or a homolog sharing at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98% or at least 99% nucleotide identity thereto. In other embodiments the inactivation is achieved by deletion of all or a part of one or more polynucleotides encoding polypeptides selected from SEQ ID NOs:12, 18 and 21 or a homolog sharing at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98% or at least 99% amino acid identity thereto. In one embodiment, the deletion is performed by using homologous recombination techniques. Homologous recombination techniques are well known to the skilled artisan. In another embodiment, the deletion is aided by using an artificial nuclease. In a further embodiment, the artificial nuclease is a Zinc finger Nuclease (ZFN) or Cas9-gRNA complex. Artificial nuclease technologies are well known to the skilled artisan. See, for example, Durai et al. (2005), Makarova et al. (2011) and Mali et al. (2013).

[0051] In one embodiment, the genetic manipulation involves over-expression of one or more polynucleotides selected from SEQ ID NOs:1, 3, 5, 7, 8, 9, 10, 11, 13, 14, 16, 17, 19 and 20, or a

homolog sharing at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98% or at least 99% nucleotide identity thereto. In another embodiment, the genetic manipulation involves over-expression of one or more polynucleotides encoding polypeptides selected from SEQ ID NOs:2, 4, 6, 12, 15, 18 and 21, or a homolog sharing at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98% or at least 99% amino acid identity thereto. In some embodiments, the over-expression is mediated by introduction of a synthetic gene cassette into a fungal host cell. In other embodiment, the cassette comprises a heterologous promoter operably linked to the polynucleotide, optionally operably linked to a transcriptional terminator. In some embodiments, the over-expression is compared to a fungal host without the genetic manipulated.

[0052] In some embodiments, the genetic manipulation involves a combination of the previously described genetic manipulations. In one embodiment, the genetic manipulation comprises the down-regulation of one or more of the polynucleotides and over-expression of one or more different polynucleotides. In another embodiment, the genetic manipulation comprises the total inactivation of enzyme function of one or more polypeptides encoded by one or more polynucleotides and the over-expression of one or more different polynucleotides. In a further embodiment, the genetic manipulation comprises the total inactivation of enzyme function of one or more polypeptides encoded by one or more polynucleotides and the down-regulation of one or more different polynucleotides. In an additional embodiment, the genetic manipulation comprises the total inactivation of enzyme function of one or more polypeptides encoded by one or more polynucleotides, the over-expression of one or more different polynucleotides and the down-regulation of one or more different polynucleotides.

[0053] Table 1 shows representative examples genetic manipulation in fungal hosts of the present invention and the effect with respect to tuning carotenoid production or composition.

TABLE 1
Examples of Tuning Carotenoid Production or Composition

SEQ ID NO: (Gene Name)	Genetic Manipulation	Tuning Effect
1 (<i>CAR1</i>)	down-regulation	decreased level of carotenoid
3 (<i>CAR2</i>)	over-expression	increased level of carotenoid
1 (<i>CAR1</i>) 19 (<i>ROCI</i>)	over-expression down-regulation	increased level of total carotenoid

10 (<i>CCDI</i>)	deletion	increased level of torularhodin and/or its derivatives
17(<i>ALDI</i>)	over-expression	increased levels if total carotenoid, torulene and torularhodin

[0054] In some embodiments, the polynucleotides described herein have been stably incorporated in the fungal genome. In these embodiments, the polynucleotides are operatively linked to a promoter which permits efficient expression in species of the *Rhodospordium* genera and the *Rhodotorula* genera. The promoters for each incorporated polynucleotide may be the same or different. In some embodiments, the promoters are promoters found in species of the *Rhodospordium* genera and the *Rhodotorula* genera. In other embodiments, the promoters are promoters found in other fungal species. Examples of suitable promoters include, but are not limited to, promoters of the following genes encoding the following proteins: glyceraldehyde 3-phosphate dehydrogenase (GPD), acyl-CoA carrier protein (ACP), fatty acid desaturase, translation elongation factor (TEF), pyruvate decarboxylase (PDC), enolase (2-phosphoglycerate dehydratase) (ENO), peptidylprolyl isomerase (PPI), acetyl-CoA carboxylase (ACC) or transaldolase. In other embodiments, the genes described herein also include a mRNA transcriptional terminator that may be one found in any eukaryotic species and their DNA viruses.

[0055] In some embodiments, a suitable promoter is one described in International Patent Application Publication No. WO 2012/169969, incorporated by reference herein in its entirety. This published application describes several polynucleotide sequences derived from the upstream region of *glyceraldehyde phosphate dehydrogenase* gene (*GPD1*), *translation initiation factor* gene (*TEF1*), and *stearoyl-CoA-delta 9-desaturase* gene (*FAD1*) that function as promoters in fungi. The promoters described in this published application are set forth in SEQ ID NOs:94-101. In other embodiments, additional promoters are described in International Patent Application Publication No. WO 2014/142747, incorporated by reference herein in its entirety. The promoters described in this published application are set forth in SEQ ID NOs:102-118.

[0056] In addition, operable fragments of the promoter sequences described herein can be isolated using convention promoter screening assays and can be screened for efficient selection of transformed fungal cells using the techniques described herein. In one embodiment, an operable fragment, also termed a promoter portion herein, is about 400 base pairs up to about

1100 base pairs in length starting from the -1 position from the ATG codon. As used herein “up to” refers to the length of the promoter portion of the promoters set forth in the disclosed SEQ ID NOs. Thus, “up to” refers to the maximal length of the promoter sequence if less than 1100 nucleotides of the promoters of the disclosed SEQ ID NOs.

[0057] In one embodiment, a promoter sequence is provided which has at least 60% identity with any one of these promoter sequences. In another embodiment, a promoter sequence is provided which has at least 70% identity with any one of these promoter sequences. In an additional embodiment, a promoter sequence is provided which has at least 80% identity with any one of these promoter sequences. In a further embodiment, a promoter sequence is provided which has at least 90% identity with any one of these promoter sequences. In another embodiment, a promoter sequence is provided which has at least 95% identity with any one of these promoter sequences. In another embodiment, a promoter sequence is provided which has at least 98% identity with any one of these promoter sequences.

[0058] The genes to be stably incorporated into the fungal genome are typically in the form of a DNA or polynucleotide construct comprising the promoter sequences described herein, an operably linked polypeptide encoding sequence described herein and an operably linked RNA transcriptional terminator sequence. In one embodiment, any transcriptional terminator operable in species of the fungi can be used. Terminators are typically located downstream (3') of the gene, after the stop codon (TGA, TAG or TAA). Terminators play an important role in the processing and stability of RNA as well as in translation.

[0059] A DNA or nucleic acid construct that comprises a fungi operable promoter, protein encoding DNA sequence and a fungi operable terminator may also be referred to herein as an expression cassette. The expression cassette may include other transcriptional regulatory regions as are well known in the art. In other embodiments, the DNA or nucleic acid construct or expression cassette further comprises a selectable marker. Selectable markers are well known to the skilled artisan as are expression cassettes incorporating such selectable markers and promoters to drive their expression, such as described in International Patent Application Publication No. WO 2012/169969. Any suitable promoter operably linked to any suitable selectable marker can be used in the present invention. In some embodiments, one or more DNA molecules may be used in which each DNA molecule has one or more nucleic acid constructs.

[0060] In another embodiment, the present invention provides a method for producing carotenoids which comprises growing a fungal host cell described herein under conditions

suitable to produce carotenoids. Any medium with at least 5% carbon source can be used. In some embodiments, the carbon source is glucose, mannose, glycerol, sucrose, xylose or combinations thereof. In one embodiment, the medium is Medium MinCAR containing 30-100 g glucose, 1.5 g yeast extract, 0.5 g (NH₄)₂SO₄, 2.05 g K₂HPO₄, 1.45 g KH₂PO₄, 0.6 g MgSO₄, 0.3 g NaCl, 10 mg CaCl₂, 1 mg FeSO₄, 0.5 mg ZnSO₄, 0.5 mg CuSO₄, 0.5 mg H₃BO₄, 0.5 mg MnSO₄, 0.5 mg NaMoO₄ (per liter). The medium is preferably adjusted to pH 5-7. In some embodiments the cell culturing is preferably performed at 25°-35°C. In other embodiments, the culturing is preferably performed in a condition with lighting.

[0061] In preparing the nucleic acid construct or an expression cassette, the various DNA fragments may be manipulated, so as to provide for the DNA sequences in the proper orientation and, as appropriate, in the proper reading frame. Toward this end, adapters or linkers may be employed to join the DNA fragments or other manipulations may be involved to provide for convenient restriction sites, removal of superfluous DNA, removal of restriction sites, or the like. For this purpose, *in vitro* mutagenesis, primer repair, restriction, annealing, resubstitutions, e.g. transitions and transversions may be involved.

[0062] Nucleic acids of the present invention may also be synthesized, either completely or in part, especially where it is desirable to provide plant-preferred sequences, by methods known in the art. Thus, all or a portion of the nucleic acids of the present invention may be synthesized using codons preferred by a selected host. Species-preferred codons may be determined, for example, from the codons used most frequently in the proteins expressed in a particular host species. Other modifications of the nucleotide sequences may result in mutants having slightly altered activity.

[0063] It may be useful to generate a number of individual transformed fungi with any recombinant construct in order to recover fungi free from any positional effects. It may also be preferable to select fungi that contain more than one copy of the introduced polynucleotide construct such that high levels of expression of the recombinant molecule are obtained.

[0064] The practice of the present invention employs, unless otherwise indicated, conventional techniques of chemistry, molecular biology, microbiology, recombinant DNA, genetics, immunology, cell biology, cell culture and transgenic biology, which are within the skill of the art. See, e.g., Maniatis *et al.*, 1982, *Molecular Cloning* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York); Sambrook *et al.*, 1989, *Molecular Cloning*, 2nd Ed. (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York); Sambrook and

Russell, 2001, *Molecular Cloning*, 3rd Ed. (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York); Ausubel *et al.*, 1992), *Current Protocols in Molecular Biology* (John Wiley & Sons, including periodic updates); Glover, 1985, *DNA Cloning* (IRL Press, Oxford); Russell, 1984, *Molecular biology of plants: a laboratory course manual* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.); Anand, *Techniques for the Analysis of Complex Genomes*, (Academic Press, New York, 1992); Guthrie and Fink, *Guide to Yeast Genetics and Molecular Biology* (Academic Press, New York, 1991); Harlow and Lane, 1988, *Antibodies*, (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York); *Nucleic Acid Hybridization* (B. D. Hames & S. J. Higgins eds. 1984); *Transcription And Translation* (B. D. Hames & S. J. Higgins eds. 1984); *Culture Of Animal Cells* (R. I. Freshney, Alan R. Liss, Inc., 1987); *Immobilized Cells And Enzymes* (IRL Press, 1986); B. Perbal, *A Practical Guide To Molecular Cloning* (1984); the treatise, *Methods In Enzymology* (Academic Press, Inc., N.Y.); *Methods In Enzymology*, Vols. 154 and 155 (Wu *et al.* eds.), *Immunochemical Methods In Cell And Molecular Biology* (Mayer and Walker, eds., Academic Press, London, 1987); *Handbook Of Experimental Immunology*, Volumes I-IV (D. M. Weir and C. C. Blackwell, eds., 1986); Riott, *Essential Immunology*, 6th Edition, Blackwell Scientific Publications, Oxford, 1988; Fire *et al.*, *RNA Interference Technology: From Basic Science to Drug Development*, Cambridge University Press, Cambridge, 2005; Schepers, *RNA Interference in Practice*, Wiley-VCH, 2005; Engelke, *RNA Interference (RNAi): The Nuts & Bolts of siRNA Technology*, DNA Press, 2003; Gott, *RNA Interference, Editing, and Modification: Methods and Protocols (Methods in Molecular Biology)*, Human Press, Totowa, NJ, 2004; Sohail, *Gene Silencing by RNA Interference: Technology and Application*, CRC, 2004.

EXAMPLES

[0065] The present invention is described by reference to the following Examples, which are offered by way of illustration and are not intended to limit the invention in any manner. Standard techniques well known in the art or the techniques specifically described below were utilized.

EXAMPLE 1

Strains, Chemicals, Media and Culture Conditions:

[0066] *Rhodospiridium. toruloides* strain ATCC 10657, ATCC 10788, ATCC 204091 (previous known as *Rhodotorula glutinis*), *Rhodotorula glutinis* strain ATCC 90781 were

purchased from ATCC (USA). *Rhodotorula glutinis graminis* strain WP1 and *Sporobolomyces roseus* FGSC 10293 (IAM13481) was obtained from Fungal Genetics Stock Center (University of Missouri, USA). *A. tumefaciens* strain AGL1 [33] and AGL2 [34] were used for ATMT.

[0067] *Rhodospiridium* strains were cultured at 28°-30°C in YPD broth (1% yeast extract, 2% peptone, 2% glucose) or on solid potato-dextrose agar (PDA). *A. tumefaciens* was grown at 28°C in either liquid or solid 2YT medium (1.6% tryptone, 1% yeast extract, 0.5% NaCl). *Escherichia coli* XL1-Blue was cultured in Luria-Bertani (LB) broth or on LB agar and used for routine DNA manipulation. To accumulate carotenoid and lipid production, *R. toruloides* was cultured in carotenoid producing medium (MinCAR) and lipid accumulation medium (MinRL2), respectively. MinCAR and MinRL2 was modified from the carotenoid medium [35] and lipid medium [36]. Medium MinCAR contains (per liter) 70 g glucose, 1.5 g yeast extract, 0.5 g (NH₄)₂SO₄, 2.05 g K₂HPO₄, 1.45 g KH₂PO₄, 0.6 g MgSO₄, 0.3 g NaCl, 10 mg CaCl₂, 1 mg FeSO₄, 0.5 mg ZnSO₄, 0.5 mg CuSO₄, 0.5 mg H₃BO₄, 0.5 mg MnSO₄, 0.5 mg NaMoO₄ (pH 6.1). Medium MinRL2 contains (per liter) 100 g glucose, 1.5 g yeast extract, 0.5 g (NH₄)₂SO₄, 2.05 g K₂HPO₄, 1.45 g KH₂PO₄, 0.6 g MgSO₄, 0.3 g NaCl, 10 mg CaCl₂, 1 mg FeSO₄, 0.5 mg ZnSO₄, 0.5 mg CuSO₄, 0.5 mg H₃BO₄, 0.5 mg MnSO₄, 0.5 mg NaMoO₄ (pH 6.1).

EXAMPLE 2

Isolation of Genomic DNA and Total RNA

[0068] Genomic DNA and RNA of *R. toruloides* were extracted as described previously [37]. The extracted DNA was qualified by agarose gel electrophoresis and quantified with a NanoDrop® ND-1000 Spectrophotometer (Nanodrop Technologies, USA).

EXAMPLE 3

Agrobacterium Tumefaciens-Mediated Transformation (ATMT):

[0069] Fungi transformation via ATMT was performed as described previously unless indicated otherwise [37]. The binary vectors were transformed by electroporation into *A. tumefaciens* AGL1 or AGL2 using a 0.2 mM cuvette coupled with a BioRad eletroporator set at 2.5 kV, 25 µF, 400 Ω. Transformants were selected on 2YT agar plates supplemented with streptomycin (100 µg/ml).

EXAMPLE 4

DNA Constructs

[0070] Oligonucleotides used are listed in Table 2. All restriction and modification enzymes were purchased from New England Biolabs (NEB, Massachusetts, USA).

TABLE 2
Sequences of Oligonucleotides

Name	Sequence (5'-3') (SEQ ID NO:)	Restriction Site	Description
PgpdR2-Sf	TTT <i>tactagt</i> GGACGGCTTGTCTCTCTCTG (32)	SpeI	<i>U. maydis</i> gpd1 promoter
Rt012N	TTT <i>ccatgg</i> TGAGTGATCTGGTGTGTTC (33)	NcoI	
PgpdR2-Sf	TTT <i>tactagt</i> GGACGGCTTGTCTCTCTCTG (34)		Rt <i>GPD1</i> promoter
Rt012N	TTT <i>ccatgg</i> TGAGTGATCTGGTGTGTTC (35)		
Rt127-2	GGAATCATCCGCTCGATCG (36)		Deletion of <i>CAR1</i>
Rt128-2	CAGGCCTTCGCCATCGGATT (37)		
Rt129	TCCTCTTCCGACTGGGACAA (38)		Colony PCR for <i>Δcar1</i>
Rt130	CCCAAACAACACCGAGAGGA (39)		
CAR3Lf	AAACACTGATAGTTTTTGAAGGGTGACGCACCTC (40)		Deletion of <i>CAR3</i>
CAR3Rr2	TCGAGCTCGGTACCCAGGAGGAGAAGAAGGTGATGG (41)		
Rt138	TCGCTGGATTGGTACGACAAC(42)		Colony PCR for <i>Δcar3</i>
Rt139	CCACCAGTGACCATCTCTTCG (43)		
CCD1L-Kf	AAAggtaccGACTTGTCCGAGCGAGAGAC (44)	KpnI	Deletion of <i>CCD1</i>
CCD1L-Hr	AAAaagcttAGACTCCAGAACCCGACCGTA (45)	HindIII	
CCD1R-Bf	TTTggtaccCGAGTCTCAATCCCTCCCA (46)	BamHI	
CCD1R-Str	TTTtagcctGGAGGACGGGCGATACAATC (47)	StuI	
CCD1f	GTCTTTCGCGCCCTCTTCCTC (48)		Colony PCR for <i>Δccd1</i>
CCD1r	CGTAGGAGATGACGGGCTTGC (49)		
CDS1L-Stf	TTTtagcctCTCGCTCTCCTGCACACTTCG (50)	StuI	Deletion of <i>CDS1</i>
CDS1L-Hr	TTTaagcttCGCATTTCCAGTCCCATCGC (51)	HindIII	

CDS1R-Bf	TTT ggatcc ACCCTCTACGTCCCCTTCACC (52)	BamHI	
CDS1R-Sr	TTT gagctc AACGCCTCGATCCTGACTTGC (53)	SacI	
CDS1f	GTCCTGCTCGCAACCCTCAC (54)		Colony PCR for <i>Δcds1</i>
CDS1r2	GAGACGAAGGATGGAGTGGCG (55)		
ALD1Lf	CACCCGTCCTCTCCGCTTC (56)		Deletion of <i>ALD1</i>
ALD1Rr	CCTCGCTCTTTCGCTGGTTC (57)		
Rt134	CAGCCACATTCGTTCTTCAGG (58)		Colony PCR <i>Δald1</i>
Rt135	TGGATGATGCGGATATTGAGG (59)		
Rt203Nf	TTT ccatgg AGGACACTCCCATCGACAGC (60)	NcoI	Expression of <i>ALD1</i>
Rt204Br	TTT ggatcc CCTGTCCCGTCAACTTCTGC (61)	BamHI	
CARSL-Stf	TTT aggcct CAGCCAAGTTCAAGCACAACC (62)	StuI	Deletion of <i>ROCI</i>
CARSL-Hr	TTT aatgctt CGACCGATCTCGAGGAGACAT (63)	HindIII	
CARSR-Bf	AA agatcc GGAACGATACCCTCCAAGACG (64)	BamHI	
CARSR-Sr	AA agagctc TGGGAGTTGCGAGGTCATAGA (65)	SacI	
CARSf	TTGTTCTCGGATGTGCGATTGG (66)		Colony PCR for <i>Δroc1</i>
CARSr	ATAATCTTGGTGAGCGCGATGTT (67)		
Rt301Nf	TTT ccatgg CGACTCTAGCCATCAGACC (68)	NcoI	Expression of <i>ROCI</i>
Rt302Evr	TTT gatatc GAGGCTAGGCGATGTTGCAG (69)	EcoRV	
Rt303Sf	TTT actagt CAAGATCTACGAGGCGAC (70)	SpeI	Complementation of <i>ROCI</i>
Rt304Pmr	TTT gtttaaac GAGTGCCCAACGACTTTCTAC (71)	PmeI	
Rt140	CGCTGACCTTCCCAATCTTTC (72)		DIG-probe for <i>CAR1</i>
Rt141	CTTTCCGACCGACTTCTTGCT (73)		
Rt146	GAACCGCAGGTGAAGGTCAAT (74)		DIG-probe for <i>CAR3</i>
Rt147	TATCGGCAAGGTACGTCTCTCTTC (75)		
Rt148	CAGGTTTCATCGCAACTACATTGA (76)		DIG-probe of <i>ALD1</i>
Rt149	AACAGAGCGAGTTGAAGAGTAGCC (77)		
qCAR3f	GCGACGACTACGTGAACCTG (78)		qPCR of <i>CAR1</i>
qCAR3r	CGATGGGGAAGGAGAATTTG (79)		

qCAR2f	GCACACTGCACGCCTTACTC (80)		qPCR of <i>CAR2</i>
qCAR2r	ACGAGCTGAAGAGCCTGTCC (81)		
qCAR1f	GCAAGATACCCCAGCTCGAC (82)		qPCR of <i>CAR1</i>
qCAR1r	GGGGACGTTGACGTAGAAGG (83)		
qCCD1f	GGCTGGATGAAGGAGTGGAC (84)		qPCR of <i>CCD1</i>
qCCD1r	AGGAGGAGCGTGAGTGGAAG (85)		
qCDS1f	ATGGGACTGGAAATGCGAAC (86)		qPCR of <i>CDS1</i>
qCDS1r	GGGAGACGAAGGATGGAGTG (87)		
qALD1f	TCGTGCACAACCCGAACACTAC (88)		qPCR of <i>ALD1</i>
qALD1r	ATCTTGCGCTCCTTCTCGTC (89)		
qROC1f	ACCAGCTTCAGACCACGTCTC (90)		qPCR of <i>ROC1</i>
qROC1r	AGAAGTTGGAGGAAGGGATGG (91)		
qACT1f	CGACAACTTTGACGACCCTTC (92)		qPCR of <i>ACT1</i>
qACT1r	CAGGTTGGGACAAGTTGGGTA (93)		

[0071] Various promoters, such as promoter of *U. maydis gpd1* (*Pgpd*, 595 bp in length) [38, 39] and *RtGPD1* (795 bp) [37], have been described previously and was amplified using the template of plasmid pEX1 [42] and genomic DNA of *R. toruloides* ATCC 10657, using primer pairs *Pgpd*-Sf/*Pgpd*-Nr and *Rt011S*/*Rt012N*, respectively. The resultant PCR products were digested with *SpeI* and *NcoI* and cloned by 3-fragment ligation with the 1030-bp *BspHI*/*SmaI* DNA fragment of synthetic *hpt-3* gene [37] and the 8855-bp *SpeI*/*SacI* (blunt-ended) DNA fragment of pEC3GPD-GUS, creating pEC3GPD-HPT3 and pEC3GPDR-HPT3, respectively.

[0072] To create deletion constructs for *CAR1*, *CAR3* and *ALD1*, the DNA fragment covering complete coding regions of the gene (3.0 kb, 2.8 kb and 3.0 kb, respectively) was amplified using genomic DNA of *R. toruloides* ATCC 10657 as the template and oligo pair *Rt127-2*/*Rt128-2*, *CAR3Lf*/*CAR3Rr2* and *ALD1Lf*/*ALD1Rr* as primer pairs, respectively. The blunt-ended PCR product was ligated to *PmeI*/*SacI* (blunt-ended) pEX2 vector to create the intermediate vector pEX2CAR1, pEX2CAR3 and pEX2ALD1, respectively. Subsequently, the hygromycin resistance cassette (*RtGPD1::hpt-3::Tnos* with the 795 bp version of *RtGPD1* promoter driving the expression of *hpt-3*) amplified from plasmid pRH2034 was ligated to the *SmaI*/*MfeI*-cut pEX2CAR1, *PvuII*/*BglII*-cut pEX2CAR3, and *XhoI*/*BspHI*-cut pEX2ALD1

(blunt ended) to create gene targeting plasmid, pKOCAR1, pKOCAR3 and pKOALD1, respectively.

[0073] For deletion of *CCD1*, the right and left arm (0.9 kb each) for homologous recombination was amplified using genomic DNA of *R. toruloides* ATCC 10657 as the template and specific oligo pair CCD1L-Kf/CCD1L-Hr and CCD1R-Bf/CCD1R-Str, respectively. Gene deletion plasmid pKOCCD1 was created by four-fragment ligation consisting of *KpnI*/*HindIII*-digested right arm, *BamHI*/*StuI*-digested left arm, *HindIII*/*BamHI* hygromycin resistance cassette from pDXP795hptR [32] and *KpnI*/*SacI*-digested pEX2 vector. Recombinant *E. coli* strains with the correct fragments were identified by colony PCR followed by DNA sequencing of the entire recombination cassette. A similar strategy was applied for the deletion of *CDS1*. Oligo pairs CDS1L-Stf/CDS1L-Hr and CDS1R-Bf/CDS1R-Sr was used to amplify the right and left homolgy arms of *CDS1* (0.5 kb each), respectively. The DNA fragments were digested using *StuI*/*HindIII* for the left arm and *BamHI*/*SacI* for the right arm, which were ligated with the *HindIII*/*BamHI* hygromycin resistance cassette and *SmaI*/*SacI*-digested pEX2 vector.

[0074] For *in vitro* expression of *ALD1*, cDNA sequences were amplified by RT-PCR with the template of total RNA and oligos Rt203Nf and Rt204Br. The *NcoI*-*BamHI* double digested PCR products were cloned in pRH2034 vector at the same sites to create the plasmid pRHALD1, in which a fusion Ald1-eGFP was driven by the *RtGPD1* promoter.

[0075] For deletion of *ROCI*, oligo pairs CARSL-Stf/CARSL-Hr and CARSR-Bf/CARSR-Sr were used to amplify the 5' and 3' homologous flanking fragments (0.9 kb each). A four-fragment ligation was performed with *SacI*-*PmeI* pEX2 binary vector, *StuI*-*HindIII* 5'flanks, *BamHI*-*HindIII* codon-optimized hygromycin resistant cassette from pDXP795hptR (*P_{GPD1}::hpt-3::T_{nos}*) and *BamHI*-*SacI* 3'flanks to generate gene deletion plasmid pKOROC1, where *P_{GPD1}* is the glyceraldehyde 3-phosphate promoter of *R. toruloides* ATCC 10657 with GenBank accession number of JN208861, *hpt-3* is the codon-optimized gene encoding hygromycin phosphotransferase (JQ806387), and *Tnos* is the terminator of agrobacterium (Liu, Koh et al. 2013).

[0076] For complementation studies of *Δcar1*, the *CAR1* genome locus ranging from 389556 to 393649 nt of WGS scaffold#18 was amplified using the template of *R. toruloides* and oligos Rt127-2 and CDSL1. The 4.1 kb PCR products were blunt-ended and ligated with *SpeI* (blunt end)-*PmeI*-linearized pRH2034 vector to create the complementation plasmid pRHCAR1. For complementation studies of *Δroc1*, the *ROCI* genome locus ranging from 622910 to 627480 nt of WGS scaffold#9 was amplified using the template of *R. toruloides* and oligos Rt303Sf and

Rt304Pmr. The 4.6 kb PCR products were double digested by SpeI and PmeI, and ligated with SpeI-PmeI-linearized pRH2034 vector to create the complementation plasmid pRHROC1.

EXAMPLE 5

Colony PCR and Southern Blot Analysis

[0077] Fungal colony PCR analysis was used for screening of candidate gene deletion mutants. Briefly, single colonies of transformants were cultured in 150 µl YPD broth supplemented with cefotaxime (300 mg/ml) and hygromycin (150 mg/ml) for several hours. One microlitre of cell culture were used for colony PCR analysis with appropriate oligo pair within the gene targeting region (Table 2). PCR was conducted using i-Taq polymerase (i-DNA Biotech, Singapore) and the following program: initiation at 95°C for 5 min, followed with 35 cycles of 94°C for 30 s, 58°C for 30 s and 72°C for 45 s, and further extension at 72°C for 5 min. Electrophoresis was used for identification of candidate mutants lacking of DNA fragments that could be amplified using the template of genomic DNA from WT.

[0078] To verify the true gene deletion mutants without any ectopic integration, genomic DNAs were digested with HindIII, PstI, PvuI, PvuI, HincII and PvuI for the putative knockout mutants $\Delta car1$, $\Delta car3$, $\Delta ccd1$, $\Delta cds1$, $\Delta ald1$ and Δand , respectively. DNA fragments containing the right arms of *CAR1*, *CAR3*, *ALD1*, *ROC1* and the left arms of *CCD1*, *CDS1* were labeled with digoxigenin using DIG-High prime DNA labeling and detection starter Kit II (Roche Diagnostics, USA). Southern blot hybridization was performed according to the manufacturer's instructions.

EXAMPLE 6

Quantitative Reverse Transcription PCR

[0079] Total RNA was extracted as described previously [37]. Before quantitative reverse transcription PCR (qRT-PCR), total RNA was treated with DNase I (Roche Diagnostics) to remove trace DNA and recovered by precipitation with ethanol. cDNA was synthesized using the iScript™ Reverse Transcription Supermix (Bio-Rad, USA) and q-PCR was conducted in ABI PRISM 7900HT Sequence Detection System using the ABI SYBR® Select Master Mix (Life Technologies, USA). qRT-PCR conditions were as followed: an initial 50°C for 2 min and 95°C denaturation step for 10 min followed by 40 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 1 min. Samples were analyzed in triplicates and data was acquired using the SDS 2.4 software (Life Technologies, USA). Relative gene expression levels were calculated

against the reference gene *ACT1* (SEQ ID NOs:22, 23, 24 for genomic, cDNA and protein, respectively) using the 2- $\Delta\Delta$ Ct method with the RQ Manager software v1.2.1 (Applied Biosystems, USA).

EXAMPLE 7

Screening of Genes Involved in Carotenoid Biosynthesis

[0080] *R. toruloides* haploid strain ATCC 10657 was mutagenized by random insertions of T-DNA by *Agrobacterium tumefaciens*-mediated transformation of binary T-DNA vector pRH201 (Fig. 1). Transformants were selected on YPD agar medium supplemented with 300 μ g/ml cefotaxime and 150 μ g/ml hygromycin. After incubated at 28°C for 5 days, transformants showing albino or pale colors were transferred to liquid YPD medium (300 μ g/ml cefotaxime, 150 μ g/ml hygromycin) for propagation. After streaking on PDA plates supplemented with above antibiotics, single colonies showing stable color phenotype were named as *Rhodospiridium* Carotenoid Mutant (RCM).

EXAMPLE 8

Identification of T-DNA Tagging Positions

[0081] T-DNA tag positions in the genome was identified by High Efficient Thermal Asymmetric InterLaced PCR (hiTAIL-PCR) [43, 44]. Specific primers (HRSP1, HRSP2 and HRSP3) and arbitrary primer LAD1-4 were used for T-DNA left border (LB) flanking sequences whereas specific primers (HRRSP1, HRRSP2 and HRRSP3) and arbitrary primer LAD1-4 for the right border (RB) flanking sequences. PCR reactions were carried out with i-Taq DNA polymerase (i-DNA, Singapore) in a PTC-200TM Programmable Thermal Controller (Bio-Rad, USA). PCR products were purified using gel extraction kit (Qiagen, USA) and sequenced directly using BigDye terminator kit (Applied Biosystems, USA) with oligo HRRSP3 (for RB-flanking sequences) or HRSP3 (LB-flanking sequences). For samples that gave poor sequencing results, PCR products were cloned in pGTM-T easy vector (Promega, USA) and sequenced using oligos M13FP and M13RP.

EXAMPLE 9

Extraction of Carotenoids

[0082] Cells were cultured in 50 ml MinCAR medium in shaking flasks at 30°C and pelleted by centrifugation. After washing twice with water, wet cell mass were determined by weighing

and mixed with equal mass of acid-washed glass beads (0.4-0.6 mm in diameter, Sigma-Aldrich) and 5 ml DMSO. Cells were lysed by vigorous vortex mixing for 10 min, 1 h incubation at 65°C followed by freezing at -20°C. After thawing, the suspension was centrifuged at 10,000 g, and the supernatant containing DMSO-soluble carotenoids was transferred to a new tube while the insoluble cell residue was re-extracted with 30 ml of light petroleum ether-ethyl acetate (36:19) for 10 min at room temperature. The contents of the two extractions were combined and extracted with 2 ml saturated NaCl. The solvent phase was collected after centrifugation and dried under a nitrogen gas flow. The samples were re-dissolved in hexane and stored in -20°C before further analysis.

EXAMPLE 10

Quantification Methods

[0083] Cell biomass (dry cell weight) was determined by lyophilizing the cell pellet collected by centrifugation until a constant weight was reached.

[0084] Glucose concentration in media was quantified by HPLC. Medium was separated from cells by centrifugation and filtered through a 0.2 µm nylon membrane. 10 µl of the sample was injected and run through a 300 x 7.0 mm Aminex 87H column (Bio-Rad) at a constant flow rate of 0.7 mL/min using 5 mM sulfuric acid as the mobile phase. The column was maintained at 50°C, and glucose was detected with a Refractive Index Detector (RID, Shimadzu, Japan). Concentration of glucose in the cell culture was determined using a calibration curve built with the standard glucose aqueous solution.

[0085] The major peaks were determined by the absorption spectra in hexane and mass spectrometry. Atmospheric pressure chemical ionization (APCI) technique is as described previously [46, 47] with some modifications. Briefly, samples (x µl) was run in a Shimadzu UPLC-MS (APCI) system (Shimadzu, Japan) equipped with a YMC-carotenoid column (C30 reverse phase, Φ3 µm, 150 mm x 3.0 mm I.D., YMC, Japan) at a flow rate of 0.3 ml/mL in a linear gradient within 3 min, from 100% mixture A (MeOH/tert-butylmethylether/water, 30:1:10, v/v/v) to 50% mixture B (MeOH/tert-butylmethylether, 1:1, v/v) followed with 100% mixture B for 0.5 min and then the column was maintained under the conditions for 15 min at a flow rate of 0.6 ml/min. APCI in positive mode was used for the identification of carotenoid components with 15 L/s of nitrogen gas as sheath and auxiliary gas. The vaporizer and capillary temperature was set at 350°C and 150°C, respectively, and the capillary and tube lens voltages was set at 50 V and 125 V, respectively.

EXAMPLE 11

Characterization of Carotenoid Biosynthetic Mutants

[0086] Screening of about 20,000 T-DNA transformants by visual identification of colony colors lead to the identification of six carotenoid mutants, which are named RAM1-5 (*R. toruloides* Carotenoid Mutants) (Figs. 2a-2E, Table 3). HiTAIL PCR and BLAST search of the sequence tags revealed that T-DNAs were inserted into genes encoding a putative riboflavin transporter, aldehyde hydrogenase, hexose transporter, TATA-binding protein associated factor, phytoene desaturase and fatty aldehyde dehydrogenase respectively (Table 3). The role of phytoene desaturase, or Car1 in fungi (EC 1.3.99.30) in lycopene production is well known [48].

TABLE 3

Characterization of *R. toruloides* Albino Mutants (RAM)

Sequence number ^a	Genic site ^{b,c}	Best hit ^d	Annotation ^e	Organism ^f	Identity ^g
RB sequences					
RAM1	Genic sequence	XP_003032296	Riboflavin transporter MCH5	<i>Schizophyllum commune</i>	52%
RAM2	Upstream-0.5 kb	YP_001220603	resolvase	<i>Aeromonas bestiarum</i>	95%
RAM3	Genic sequence	XP_571856	hexose transport-related protein	<i>Cryptococcus neoformans</i>	36%
RAM4	Genic sequence	XP_758766	TATA-binding protein (TBP) associated factor Taf2 (MTCC 457 contig458_1:18376-18377+)	<i>Ustilago maydis</i>	35%
RAM5	Genic sequence	KF601426.1	phytoene synthase	<i>Rhodospiridium diobovatum</i>	98%

^a Flanking sequence obtained from corresponding to number of T-DNA transformant

^b T-DNA tagged genes were determined according to the BLASTx results

^c Upstream 1.0 kb, Upstream 0.5 kb and downstream 0.3 kb denotes T-DNA insertions within upstream 501~1000 bp, 500 bp and downstream 300 bp of the corresponding tagged gene, respectively

^d Best hit denotes the BLASTx result with the highest E-score

^e Annotations were determined according to the BLASTx results

^f Microorganism denotes the host of Best hit

^g Identity values were from BLASTx results

^h Not available due to the bad sequencing result

EXAMPLE 12

Analysis of Carotenoid Biosynthesis Gene Cluster in *R. toruloides* by Reverse Genetics

[0087] HiTAIL PCR revealed that the RCM5 albino phenotype resulted from a T-DNA inserted between 391802 nt and 391803 nt in genome scaffold #18 (AEVR02000018) of *R. glutinis* ATCC 204091, located in the 3rd exon of the putative phytoene desaturase gene (*CAR1*, genome locus RTG_00274) (Fig. 2B), an enzyme involved in carotenoid biosynthesis. To confirm the function of this gene that was interrupted by the T-DNA insertion, the putative *CAR1* CDS was deleted by targeted knockout using ATMT of pKOCAR1, in which the nucleotide sequence ranging from +948 to +2097 of the CDS was replaced by the hygromycin resistant cassette ($P_{GPD1}::hpt-3::Tnos$, Fig. 2B). The correct null mutant ($\Delta car1$) was verified by Southern blot analysis (Fig. 2C). As expected, The $\Delta car1$ colony displayed a creamy color rather than the orange color observed in WT. The creamy color of $\Delta car1$ could be further restored to orange by ectopic integration of the allele of *CAR1* gene into the genome ($\Delta ene i$ strain in Fig. 2D). Analyses of carotenoid profiles confirmed the loss of β -carotene, γ -carotene, torulene and torularhodin peaks in $\Delta car1$ and all lost peaks were restored in the complemented strain, $\Delta car1C$, with the integration of T-DNA from binary vector pRHCAR1, where the whole allele of *CAR1* ranging from -1166 upstream to +517 downstream of the translational start and stop codon, respectively (Fig. 2E). Results strongly support that *CAR1* encodes one of the key enzyme involved in carotenoid biosynthesis pathway. To identify more genes in the carotenoid biosynthesis pathway, tBLASTn searches were performed using the *U. maydis* GGPP synthase (*CAR3*) (XP_760606, GenBank) and phytoene synthase/carotene cyclase (*CAR2*) (XP_762434) and the corresponding orthologous sequences in *R. toruloides* were successfully identified. *CAR3* CDS was found located in nt 849806-851310 in scaffold #13 (genome locus RTG_00457, AVER02000013) while *CAR2* in nt 396838-399094 in scaffold #18.

[0088] We have reported that *CAR2* knockout lead to albino phenotype [32]. However, details on its gene structure remained unknown. Using rapid amplification of cDNA ends (RACEs) and reverse transcription PCR (RT-PCR) techniques, cDNA sequences of for *CAR1* (SEQ ID NO:1), *CAR2* (SEQ ID NO:3), and *CAR3* (SEQ ID NO:5) were obtained. The *CAR1*, *CAR2* and *CAR3* cDNAs spans 2430, 2334, and 1546 genomic nt in length, containing 10, 8 and 6 exons and encode proteins of 554 (Car1, SEQ ID NO:2), 608 (Car2, SEQ ID NO:4) and 359 (Car3, SEQ ID NO:6) aa, with 19, 77 and 41 nt 5'UTR in the cDNAs, respectively. The

corresponding genomic sequences are listed in SEQ ID NOs:7, 8 and 9, respectively. The splicing of the 3 mRNAs strictly follows the canonical GU-AG rule. The results also revealed that the T-DNA of RAM5 was integrated into between +681 and +682 from the start codon of *CAR1*, resulting in premature termination of *CAR1* mRNA translation after the 158th aa (Fig. 2B).

[0089] *CAR1* and *CAR2* are located in the same scaffold #18, separated by a 4354 bp DNA sequence. This organization is analogous to several other carotenogenic fungi, such as *Blakeslea trispora*, *Fusarium fujikuroi*, *Phycomyces blakesleeanus* and *Sporobolomyces roseus* [49-52] (Fig 3A). A homologous search (BLASTx, NCBI) of the DNA sequence between the genomic locus *CAR1* and *CAR2* uncovered two putative genes encoding a carotenoid cleavage dioxygenase (Ccd1) and a carotenoid desaturase (Cds1) (Fig. 3A). 5' and 3' RACEs and RT-PCR revealed that *CCD1* (SEQ ID NO:11) and *CDS1* (SEQ ID NO:14) cDNAs span 2079 and 793 genomic nt in length, containing 4 and 3 exons with 41 and 19 nt 5'UTR encoding 636 (Ccd1, SEQ ID NO:12) and 224 aa (Cds1, SEQ ID NO:14) proteins, respectively. Again, the splicing strictly follows the canonical GU-AG rule. The corresponding genomic sequences are listed in SEQ ID NOs:10 and 13, respectively.

[0090] The divergent organization of *CAR1* and *CAR2* are analogs to *Mucor circinelloides* (scaffold#1), *Phycomyces blakesleeanus* (scaffold#5), *Blakeslea trispora* and *F. fujikuroi* (chromosome#11). except that *CCD1* and *CDS1* gene are found only in *R. toruloides*. *S. roseus* genome appeared to have undergone a recombination between *CAR1* and *CAR2*, resulted in loss of *CDS1* and translocation of *CAR1* (Fig. 5A). *F. fujikuroi* *CarX* is located outside of the *CarRA* (*CAR2* ortholog) and *CarB* (*CAR1* ortholog) and considered as the ortholog of *CCD1* because its protein product exhibits highly aa sequence homologous to Ccd1 (43% identity). The genetic synteny shared among these carotenogenic fungi suggests a common evolutionary origin of the carotenoid cluster genes.

[0091] To confirm their functions in carotenoid biosynthesis, null mutants were created. Similar to $\Delta car1$ and $\Delta car2$, $\Delta car3$ colonies exhibited a creamy color phenotype while $\Delta ccd1$ and $\Delta cds1$ also showed significantly different colors to WT (Fig. 3C). HPLC analysis of their carotenoids revealed totally abolished carotenoid production in either $\Delta car3$, $\Delta car2$ and $\Delta car1$ (data not shown). Total carotenoid production levels were slightly increased in $\Delta ccd1$ by 18% but dramatically decreased in $\Delta cds1$ by 69% (Fig. 3D). Except the slight decrease in β -carotene (6%), regarding to the components of carotenoids accumulated, deletion of *CCD1*

could result in 86%, 14% and 65% increase in torularhodin, torulene and γ -carotene, respectively (Fig. 3D). In *cds1* mutant, the quantitation of all carotenoid components were decreased, especially more than half decreases in torularhodin and torulene (Fig. 3D).

EXAMPLE 13

Effect of ALD1 Deletion on Carotenoid Production

[0092] The RCM6 mutant was initially identified in a screening for genes that affect fatty acid biosynthesis and the representative gene *ALD1*, a fatty aldehyde dehydrogenase encoding gene, was found to be involved in the degradation of polyunsaturated fatty acid (alpha-linolenic acid, C18:3n=9) (U.S. provisional patent application No. 62/047,300 filed on 8 September 2014, incorporated herein by reference). Regarding to the significant difference in cell color against WT, *ALD1* was also studied for the role in carotenoid biosynthesis. The gene deletion mutant $\Delta ald1$ was generated by homologous recombination through ATMT using the binary vector pKOALD1. To obtain the gene overexpression mutant, the Ald1-RtGFP fusion protein was fused to RtGPD1 promoter and ectopically integrated into $\Delta ald1$ through ATMT (OEALD1 mutant). After a 5-day fermentation in the carotenoid-producing medium MinCAR, the total carotenoid yield in $\Delta ald1$ was similar to WT, however beta-carotene level was increased by about 36% while. In contrast, both torulene and torularhadin levels were significantly increased in the ALD1 over expression strain (Fig. 4). A genomic sequence, cDNA sequence and protein sequence for ALD1 are set forth in SEQ ID NOs:16, 17 and 18, respectively.

EXAMPLE 14

mRNA Profiles of *CAR1*, *CAR2*, *CAR3*, *ALD1*, *CCD1* and *CDS1*

[0093] Single colonies of *R. toruloides* ATCC 10657 and carotenoid mutants were inoculated into 50 ml YPD broth in 250 ml conical flasks and cultured at 28°C and 250 rpm till saturation. The cell cultures were separated in half and cultured continued for each in a shaking platform for 5 more hours, where illumination was conducted using a fluorescent light (Philips TLD 36 W/865, 4 W/m² white light, 75 cm away) or avoided by covering with aluminum foil. Cells were sampled at various time points and total RNAs were extracted. qRT-PCR was performed in triplicates using oligo pairs qCAR3f/qCAR3r, qCAR2f/qCAR2r, qCAR1f/qCAR1r, qCCD1f/qCCD1r, qCDS1f/qCDS1r and qALD1f/qALD1r for *CAR3*, *CAR2*, *CAR1*, *CCD1*, *CDS1*, *ALD1*, respectively (Table 2). Relative mRNA levels were calculated against the reference gene actin (oligo pair qACT1f/qACT1r, Table 2) using the 2- $\Delta\Delta C_t$ method. As shown

in Fig. 5, mRNA levels of *CAR1* and *CAR2* were much lower than that of *CAR3* and were likely the bottleneck for the redirection of carbon flux to carotenoid production. *CAR1*, *CAR2*, *CAR3* and *ALDI* mRNA were light inducible.

EXAMPLE 15

Characterization of *ROC1*

[0094] In *Fusarium fujikuroi*, *carS* disruption showed an enhanced carotenoid biosynthesis irrespective of light illumination (Avalos and Cerdà-Olmedo 1987). However, genes involved in the earlier steps of terpenoid biosynthesis pathway such as FPP synthase and HMGR, were not affected by the deletion of *carS* (Rodriguez-Ortiz, Limon et al. 2009).

[0095] Using *Fusarium fujikuroi* *carS* protein sequence (NCBI GenBank accession number CCP50075.1) as query to search against the whole-genome shotgun sequences of *R. glutinis* ATCC 204091 through the online program tBLASTn (NCBI, USA), a putative ortholog was found in sequence scaffold#9 (E-value and query cover of 4E-37 and 53%, respectively). To be consistent with the gene nomenclature of *R. toruloides* putative gene was termed *ROC1* (Regulator of carotenoid biosynthesis).

[0096] 5' and 3' RACE yielded a pair of cDNA fragments of approximate 0.9 and 0.7 kb for *ROC1* (data not shown). Using oligo pair Rt301Nf and Rt302Evr (Table 2), the full-length cDNA of *ROC1* could successfully amplified by RT-PCR (data not shown). Comparison between the cDNA and genomic sequences revealed 5 exons for *ROC1* (Fig. 6A), where the splicing junctions abide strictly to the conical GU-AG rule.

[0097] *ROC1* spans a 3136-nt genome region (SEQ ID NO:19) encoding a mRNA of 2805 nt with a 84 nt 5'UTR (SEQ ID NO:20). *ROC1* encodes a 934-aa protein (SEQ ID NO:21) containing a RING-finger domain (C3HC4 type, NCBI conserved domain cd00162) and an ATP-dependent protease La (LON) domain (pfam02190). The sequence shows 96% and 97% aa-identity to a homolog in *R. toruloides* strain NP11 (EMS19961.1) and CECT1137 (CDR44527.1, respectively).

[0098] *ROC1* shares less than 20% identities to orthologs of various carotenogenic fungi except those of two zygomycete, *B. trispora* and *M. cricineloides* (Fig. 6B). Furthermore, the protein sequences within the core RING-finger domain also show very low homology, some even lacking the C3HC4 motif (Fig. 6C).

EXAMPLE 16

Effects of Deletion of *ROC1*

[0099] To genetically understand the role of *ROC1* on carotenoid biosynthesis, the *ROC1* gene was deleted through homologous recombination (Fig. 7A). Transformation with the gene deletion vector (pKOROC1) showed two kinds of transformants with obvious color differences, light and deep orange (Fig. 7A), and the deep orange transformants were found to be the true knockouts by Southern blot analysis (Fig. 7B). *roc1* null mutants showed similar cell morphology and growth to WT (Figs. 7C and 7D). However, significant improvement of carotene biosynthesis could be observed in *roc1* mutants and this could be completely restored by introduction of a WT *ROC1* gene allele in *roc1* mutant (Fig. 7D). Surprisingly, cell biomass production was significantly decreased in the complementation mutant (Fig. 7D). Quantitation analysis revealed that the 5-day culture in MinCAR medium yielded about 1.5-fold more carotenoids in *roc1* mutant as compared to those in WT (Fig. 7E).

[0100] The use of the terms “a” and “an” and “the” and similar referents in the context of describing the invention (especially in the context of the following claims) are to be construed to cover both the singular and the plural, unless otherwise indicated herein or clearly contradicted by context. The terms “comprising,” “having,” “including,” and “containing” are to be construed as open-ended terms (i.e., meaning “including, but not limited to,”) unless otherwise noted. Recitation of ranges of values herein are merely intended to serve as a shorthand method of referring individually to each separate value falling within the range, unless otherwise indicated herein, and each separate value is incorporated into the specification as if it were individually recited herein. All methods described herein can be performed in any suitable order unless otherwise indicated herein or otherwise clearly contradicted by context. The use of any and all examples, or exemplary language (e.g., “such as”) provided herein, is intended merely to better illuminate the invention and does not pose a limitation on the scope of the invention unless otherwise claimed. No language in the specification should be construed as indicating any non-claimed element as essential to the practice of the invention.

[0101] Embodiments of this invention are described herein, including the best mode known to the inventors for carrying out the invention. Variations of those embodiments may become apparent to those of ordinary skill in the art upon reading the foregoing description. The inventors expect skilled artisans to employ such variations as appropriate, and the inventors intend for the invention to be practiced otherwise than as specifically described herein.

Accordingly, this invention includes all modifications and equivalents of the subject matter recited in the claims appended hereto as permitted by applicable law. Moreover, any combination of the above-described elements in all possible variations thereof is encompassed by the invention unless otherwise indicated herein or otherwise clearly contradicted by context.

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WHAT IS CLAIMED IS:

1. A method for tuning the production level and composition of carotenoids in a fungal host comprising:
 - (a) genetically manipulating one or more polynucleotides in carotenoid biosynthesis in a fungal host, wherein the one or more polynucleotides are selected from (i) the polynucleotides set forth in SEQ ID NOs:1, 3, 5, 7, 8, 10, 11, 13, 14, 16, 17, 19 and 20 or a homologous sequence sharing at least 75% identity thereto or (ii) one or more polynucleotides encoding one or more polypeptides set forth in SEQ ID NOs:2, 4, 6, 12, 15, 18 and 21 or a homologous sequence sharing at least 75% identity thereto, and wherein the fungal host is *Rhodospordium* or *Rhodotorula*, and
 - (b) growing the fungal host to produce carotenoids, wherein the carotenoids are selected from the group consisting of lycopene, beta-carotene, gamma-carotene, torulene and torularhodin or derivatives thereof,whereby the production level or composition of the carotenoids is tuned.
2. The method of claim 1, wherein the genetic manipulation is down-regulation of the one or more polynucleotides, wherein the down-regulation is compared to a fungal host not having the genetic manipulation.
3. The method of claim 2, wherein the one or more polynucleotides are down-regulated by RNAi, an artificial transcriptional repressor or a weak promoter.
4. The method of claim 1, wherein the genetic manipulation is over-expression of the one or more polynucleotides, wherein the over-expression is compared to a fungal host not having the genetic manipulation.
5. The method of claim 4, wherein the one or more polynucleotides are over-expressed by introducing one or more DNA molecules into the fungal host, wherein each DNA molecule comprises one or more constructs and wherein each construct comprises a heterologous promoter operatively linked to one of the polynucleotides operatively linked to a transcriptional terminator.

6. The method of claim 1, wherein genetic manipulation is the total inactivation of one or more enzyme functions of one or more polypeptides encoded by the one or more polynucleotides.
7. The method of claim 6, wherein the genetic manipulation is the deletion of all or a part of one or more of the polynucleotides, wherein the one or more polynucleotides are selected from (i) the polynucleotides set forth in SEQ ID NOs:10, 11, 16, 17, 19 and 20 or (ii) one or more polynucleotides encoding one or more polypeptides set forth in SEQ ID NOs:12, 18 and 21.
8. The method of claim 7, wherein the deletion is made by homologous recombination.
9. The method of claim 7, wherein the deletion is made using an artificial nuclease selected from a Zinc finger nuclease or a Cas9-gRNA complex.
10. The method of claim 2 or 3, wherein the genetic manipulation further includes over-expression of one or more different polynucleotides than the one or more polynucleotides down-regulated, wherein the over-expression is compared to a fungal host not having the genetic manipulation.
11. The method of claim 10, wherein the one or more polynucleotides are over-expressed by introducing one or more DNA molecules into the fungal host, wherein each DNA molecule comprises one or more constructs and wherein each construct comprises a heterologous promoter operatively linked to one of the polynucleotides operatively linked to a transcriptional terminator.
12. The method of any one of claims 6 to 9, wherein the genetic manipulation further includes over-expression of one or more different polynucleotides than the one or more polynucleotides encoding the one or more polypeptides having loss of enzyme function, wherein the over-expression is compared to a fungal host not having the genetic manipulation.

13. The method of claim 12, wherein the one or more polynucleotides are over-expressed by introducing one or more DNA molecules into the fungal host, wherein each DNA molecule comprises one or more constructs and wherein each construct comprises a heterologous promoter operatively linked to one of the polynucleotides operatively linked to a transcriptional terminator.
14. The method of any one of claims 6 to 9, wherein the genetic manipulation further includes down-regulation of one or more different polynucleotides than the one or more polynucleotides encoding the one or more polypeptides having loss of enzyme function, wherein the down-regulation is compared to a fungal host not having the genetic manipulation.
15. The method of claim 14, wherein the one or more polynucleotides are down-regulated by RNAi, artificial transcriptional repressor or weak promoter.
16. The method of claim 12 or 13, wherein the genetic manipulation further includes down-regulation of one or more different polynucleotides than the one or more polynucleotides encoding the one or more polypeptides having loss of enzyme function or the one or more polynucleotides that are over-expressed, wherein the down-regulation is compared to a fungal host not having the genetic manipulation.
17. The method of claim 16, wherein the one or more polynucleotides are down-regulated by RNAi, artificial transcriptional repressor or weak promoter.
18. The method of any one of claims 1 to 17, wherein the fungal host is grown in a medium comprising at least 5% of a carbon source at a temperature from about 25°C to about 35°C, preferably under illumination.
19. The method of claim 18, wherein the carbon source is glucose, mannose, glycerol, sucrose, xylose or combinations thereof.
20. The method of claim 18 or 19, wherein the medium comprises 30-100 g/L glucose, 1.5 g/L yeast extract, 0.5 g/L (NH₄)₂SO₄, 2.05 g/L K₂HPO₄, 1.45 g/L KH₂PO₄, 0.6 g/L

MgSO₄, 0.3 g/L NaCl, 10 mg CaCl₂, 1 mg/L FeSO₄, 0.5 mg/L ZnSO₄, 0.5 mg/L CuSO₄, 0.5 mg/L H₃BO₄, 0.5 mg/L MnSO₄, 0.5 mg/L NaMoO₄, wherein pH of the medium is from about 5 to about 7.