Novel genetic perturbations in *Saccharomyces cerevisiae* for improving terpenoid production

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Applications and uses of terpenoids

- Flavours in foods and drinks, even toothpaste and cigarettes
Fragrances in soap, perfumes and other cosmetic products.
Taxol, a potent anticancer agent produced by *Taxus* plants, is a diterpene
US Patent 7,846,222 (Amyris Biotechnologies) claims a fuel composition comprising one of a group of specified isoprenoid compounds such as farnesane.
Alternative biofuels produced by cell factories

Simple sugars

**Mevalonate pathway**

Acetyl-CoA

**Bisabolene synthase**

Bisabolene 1

**Chemical hydrogenation**

Biosynthetic alternative to D2 diesel fuel

Bisabolane 2
The plants
From genes to essential oils: *Salvia fruticosa*
Mining the genes
Terpenes are produced in the glandular trichomes
Summary of EST approach (1)

Isolation of glandular trichomes from *S. fruticosa* and *S. pomifera* by mechanical means, extraction of total RNA, and construction of cDNA library

Isolation of plasmid DNA, estimation of insert size by restriction digestion and agarose gel electrophoresis, and selection

Sequencing of cDNA insert
Classification of isolated clones according to the MIPS functional classification of the closest *Arabidopsis* protein.
## Table of Hits

<table>
<thead>
<tr>
<th>Function</th>
<th>S. fruticosa</th>
<th>S. pomifera</th>
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<td><strong>Isoprene synthesis</strong></td>
<td>1-deoxy-D-xylulose 5-phosphate synthase 1</td>
<td>1-deoxy-D-xylulose 5-phosphate synthase 1</td>
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<td>farnesyl diphosphate synthase</td>
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<td>cytochrome P450 family protein 12</td>
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*aPutative is below 90% identity with a characterised protein.*
The cineole synthase active site region showing the water molecule associated with Asn 338
Sf Cineole synthase

Asn338 > Ile

Asn338 > Ile
Ala339 > Thr

Asn338 > Ile
Ala339 > Thr
Move Pro

Sp Sabinene synthase
Some substitutions of Asn338 make the pocket in the active site sufficiently to enable the farnesyl diphosphate to fit.

Do these substitutions enable synthesis of sesquiterpenes?
Sesquiterpene synthase activities resulting from one amino acid change (Asn338)

<table>
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<tr>
<th>Enzyme variant</th>
<th>cis-α-bergamotene</th>
<th>trans-α-bergamotene</th>
<th>Z-β-farnesene</th>
<th>E-β-farnesene</th>
<th>β-selinene</th>
<th>β-bisabolene</th>
<th>β-sesqui phellandrene</th>
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<td>4.0</td>
<td>49.0</td>
<td>2.6</td>
<td>16.8</td>
<td>15.1</td>
<td>9.2</td>
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<td>SfCinS1 (N338S)</td>
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<td>0.0</td>
<td>0.0</td>
<td>39.4</td>
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<td>3.1</td>
<td>17.7</td>
<td>13.5</td>
<td>10.0</td>
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Analysis of S. fruticosa transcriptome data

• The sequenced contigs were analyzed using the BlastX and PFAM algorithms.

• The output was tabulated in excel files. Manual curation identified informative homologues from other species. All contigs were transferred into the Vector NTI database. Contigs of interest relating to secondary metabolism were annotated.

• Pile-ups were performed for all terpene synthases and cytP450s
454 αλληλούχηση του μεταγραφώματος της S. fruticosa
Cytochrome P450s

Selected genes in yellow
“Metabolic Engineering” in yeast for Terpenoid Production

WHY YEAST?

• Eukaryotic organisms, they contain the same basic metabolic machinery as higher organisms
• Well established industrial microorganism from ancient times
• The first eukaryote whose genome was sequenced
• It is composed of app. 6,000 genes; collection of deletion strains available for most of them
• Additional info on yeast interactome, yeast transcriptome exists
• Abundance of molecular tools such as expression vectors, homologous recombination integration vectors
Synopsis of terpene biosynthesis in yeast indicating the genes involved and the metabolic engineering interventions employed

Acetate → ACS1, ACS2, SeACS(L641P) → Acetyl-CoA → ERG10, AtoB → Acetoacetyl-CoA → HMGS → HMG-CoA → HMG1, HMG2 → mevalonate → IPP → DMAPP → ERG20, ERG20f → GPP → FPP → GGPP → Diterpenes

Gene upregulation: ALD6, SeACS(L641P), HMG1, HMG2, iDi1, ERG20, BTS1, LPP1, DPP1
Gene downregulation: ERG9, LPP1, DPP1, GDH1

Protein engineering: HMGR, GGPPS, TPSs

sterol → squalene → Diterpenes → CPP → geranylgeraniol

Protein fusion: AtoB, Hmg, Erg20, Bts1, Dpp1, CDS, TPSs

Monoterpenes → Sesquiterpenes → Diterpenes

(®) Protein engineering, (f) fusion or scaffold attachment
Strategies for further increasing production of terpenoids in yeast

- Use of new enzymes with increased catalytic activity
- Protein stabilization
- Co-expression of terpene synthase interacting proteins which stabilize them
- Use of strong promoters
- Integration of multiple gene copies into the genome
- Intervention into competitive metabolic pathways
- Fusion proteins
- Facilitating product export
Genetic interaction in yeast

A **digenic** interaction as a double mutant that shows a significant deviation in fitness compared with the expected multiplicative effect of combining two single mutants

**Negative interactions** refer to a more severe fitness defect than expected, with the extreme case being synthetic lethality

**Positive interactions** refer to double mutants with a less severe fitness defect than expected
Positive Genetic interactors in yeast identify a new set of targets for improvement
The ERAD complex
Yeasts do it ..
In yeast diploid cells deletion of one allele, in the vast majority of cases translates into almost 50% suppression of gene expression.

Haploinsufficiency refers to a situation where a heterozygous diploid mutant is more sensitive to a stress imposed (i.e. a drug) than the wild type strains.

In our case, we aimed to identify heterozygous deletions which boost terpenoid productivity in yeast.
• Developing heterozygous deletion strains

Mat a deletion mutant

\[ \text{Mat a/alpha, } p\text{UTDH3m/Sf126 ca} \]

\[ \text{Mat a/alpha, } x/X, \ p\text{UTDH3m/SF126} \]
Solid Phase Microextraction (SPME) sampling procedure
• Screening for heterozygous deletions which enhance terpenoid productivity

WT Diploid

Gen. background
Mat a/alpha, x/X, pUTDH3m/Sf126

Gen. background
Mat a/alpha, P_{TDH3-HMG2} (K6R)::HO1, pUTDH3m/Sf126
Heterozygous deletions reproducibly increase caryophyllene yields over time

2-5 fold induction over control strain
Can we string a series of tandem heterozygous deletions in a strain?

Strategy: Select a deletion which gave consistent induction of caryophyllene production

Mate it with series of mutants to develop genetic backgrounds:

Mat a/alpha, ubc7/UBC7, x/X, pUTDH3m/Sf126
Double heterozygous and homozygous deletions in *ubc7*

In heterozygous strains, tandem deletions lead in many cases to additional yield increases.

In contrast, in double homozygous haploid strains most strains suffered serious growth and production impediments.

Selected genes: UBC7, SSM4/DOA10, PHO86
Engineered new yeast strains for high caryophyllene production

EG60-01  Mata, ura3, trp1, his3
AM94-01  Mata/a, Phis-HMG2(K6R)::HOX2, ura3, trp1, his3, Ptdh-HMG2(K6R)::leu2 X2, ERG9/erg9 Derived from AM90
AM97-01  Mata/a, Phis-HMG2(K6R)::HOX2, ura3, trp1, his3, Ptdh-HMG2(K6R)::leu2 X2, ERG9/erg9, UBC7/ubc7 derived from AM94
AM 102-01 Mata/a, Phis-HMG2(K6R)::HOX2, ura3, trp1, his3, Ptdh-HMG2(K6R)::leu2 X2, ERG9/erg9, UBC7/ubc7, SSM4/ssm4 derived from AM97
AM 109-01 Mata/a, Phis-HMG2(K6R)::HOX2, ura3, trp1, his3, Ptdh-HMG2(K6R)::leu2 X2, ERG9/erg9, UBC7/ubc7, SSM4/ssm4, PHO86/pho86 derived from AM102
Improved strains produce 50-fold higher yields of caryophyllene in yeast
Heterozygous deletions increase Hmgp stability in engineered cells
Non-targeted screen for new deletions enhancing terpenoid production

- The sesquiterpene yield increases did not coincide with diterpene productivity
- Seemingly unrelated genes and pathways could play an especially important role in the pathway efficiency
- The carotenoid biosynthetic pathway can offer an easily discernible visual phenotype which can be correlated with productivity
Plasmid construct

High-Level Production of Beta-Carotene in *Saccharomyces cerevisiae* by Successive Transformation with Carotenogenic Genes from *Xanthophyllomyces dendrorhous*Δ

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Fungal Genomics, Laboratory of Microbiology, Wageningen University, Dreijenlaan 2, 6703 HA Wageningen, The Netherlands,1 and Biosynthesis Group, Molecular Biosciences 213, J. W. Goethe Universität, Siesmayerstrasse 70, P.O. Box 111932, D-60054 Frankfurt, Germany2

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crtE : GGPP synthase

crtYB : phytoene synthase

crtI : phytoene desaturase
Plasmid digest and yeast strain transformation

Integration should eliminate plasmid instability issues during the screen

- Integration into a wild type Mat alpha yeast strain

Integration should eliminate plasmid instability issues during the screen
Mating of the Mat alpha, YEplac195-crtYB/crtI/crtE strain to the Research Genetics yeast deletion strain library

- Library (Research Genetics): 4250 deletion mutant strains
- Plating in selection media for diploid strains
Cross of library strains to Mat alpha carotenoid producing strain
Strains showing greater percentage of orange colonies or deeper red color were selected for further evaluation.
Carotenoid production in yeast as visual marker for the identification of genes participating in enhanced GGPP production
To the selected set of deletions identified, the corresponding haploid library deletion strains, the following plasmids were transformed:

- YEplac195-crtYB/crtI/crtE
- pUTDH3m/Sf126 caryoph. synthase
Μετασχηματισμός απλοειδών μεταλλαγμάτων με το πλασμίδιο YEP195

- Για να διαπιστωθεί η συνεισφορά των μεταλλάξεων στο φαινότυπο απλοειδών στελεχών

**Diploid**

**Haploid**

*mefl* strain

Dead cells

Stored carotenoids
Quantification of carotenoids

- Selected colonies were used for inoculation of 50 ml cultures
- Cultures grown after 5 days incubation period
Carotenoid production of selected haploid mutants
Caryophyllene production in selected mutants
Tandem heterozygosity can increase carotenoid yields
Current aims

• Identify tandem heterozygous mutations which boost terpenoid productivity

• Genes which do not participate in vital functions can be fully deleted

• Understand the mode of action

• Develop optimized yeast strains for diterpene production