

Novel genetic perturbations in
Saccharomyces cerevisiae for improving
terpenoid production

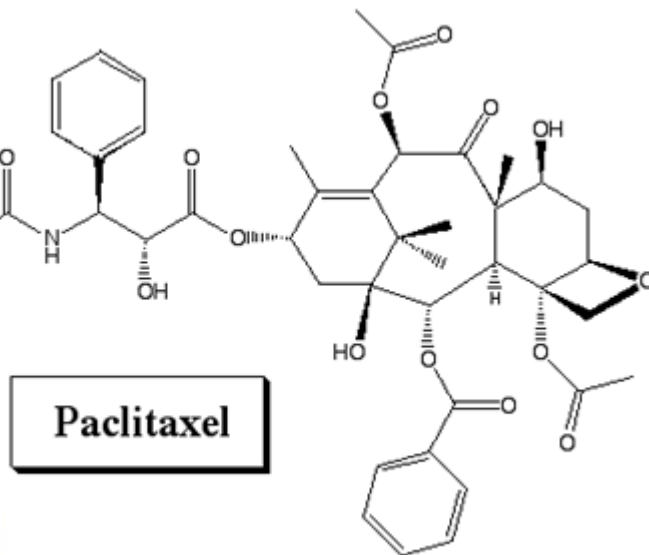
- Antonios M. Makris
  - INEB/CERTH
  - Thessaloniki
  - Greece

Applications and uses of terpenoids

- Flavours in foods and drinks, even toothpaste and cigarettes

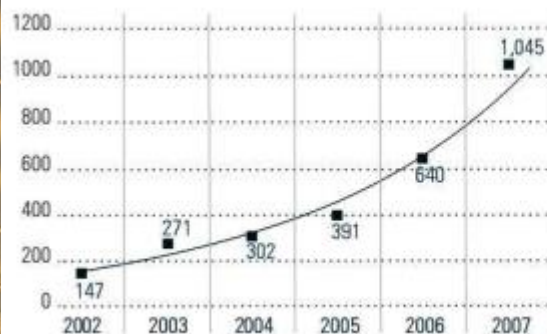


Taxol, a potent anticancer agent produced by *Taxus* plants, is a diterpene



Terpenes as biofuels

US Patent 7,846,222 (Amyris Biotechnologies) claims a fuel composition comprising one of a group of specified isoprenoid compounds such as farnesane,



Biofuel Patents from 2002 - 2007

2002 — 147 patents
 2003 — 271 patents
 2004 — 302 patents
 2005 — 391 patents
 2006 — 640 patents
 2007 — 1,045 patents

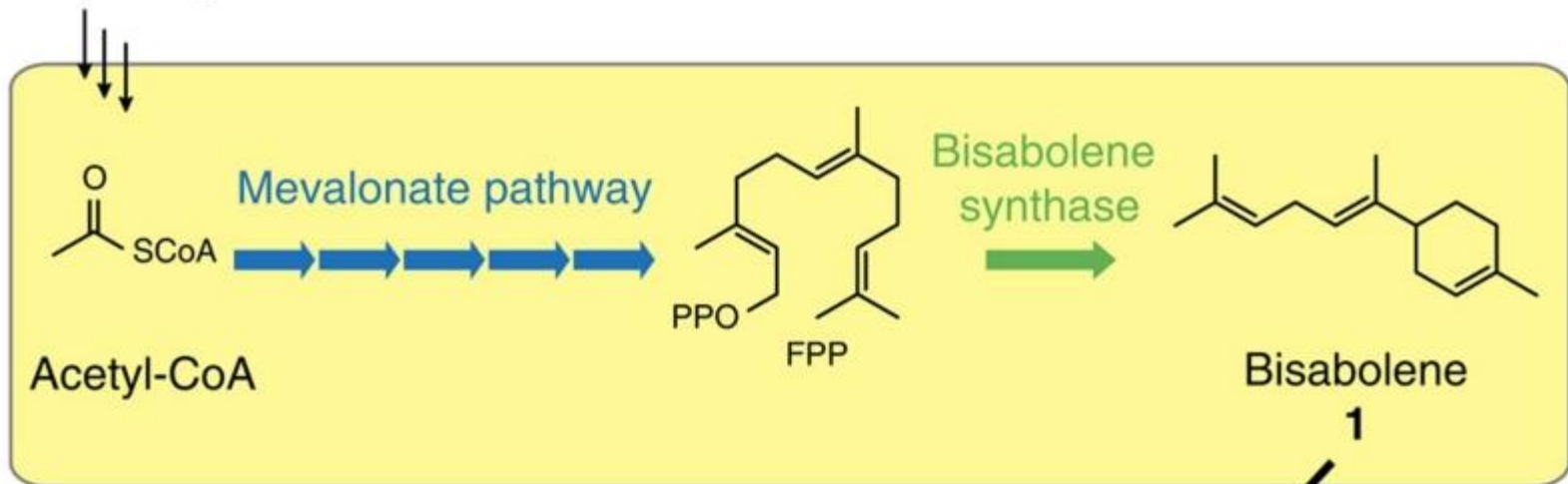
Biofuel Patents Compared to Solar Power and Wind Power Patents in 2007

■ Biofuel	1045	[56%]
■ Solar Power	555	[29%]
■ Wind Power	282	[15%]

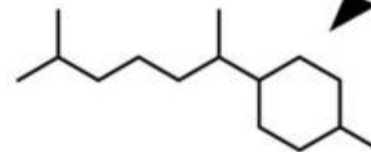


Alternative biofuels produced by cell factories

Simple sugars



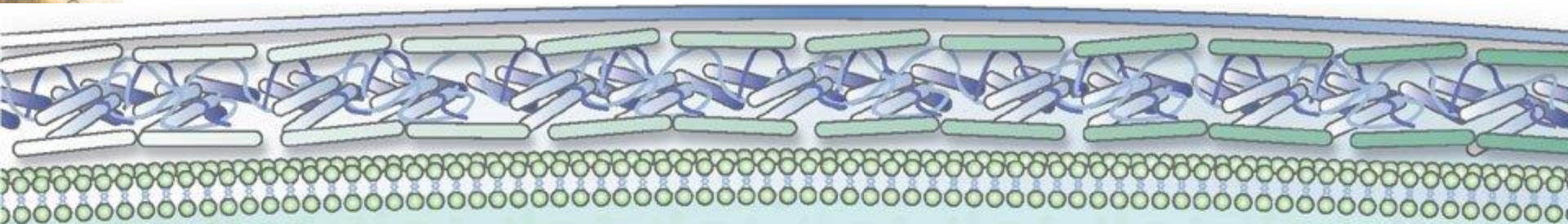
Biosynthetic alternative
to D2 diesel fuel



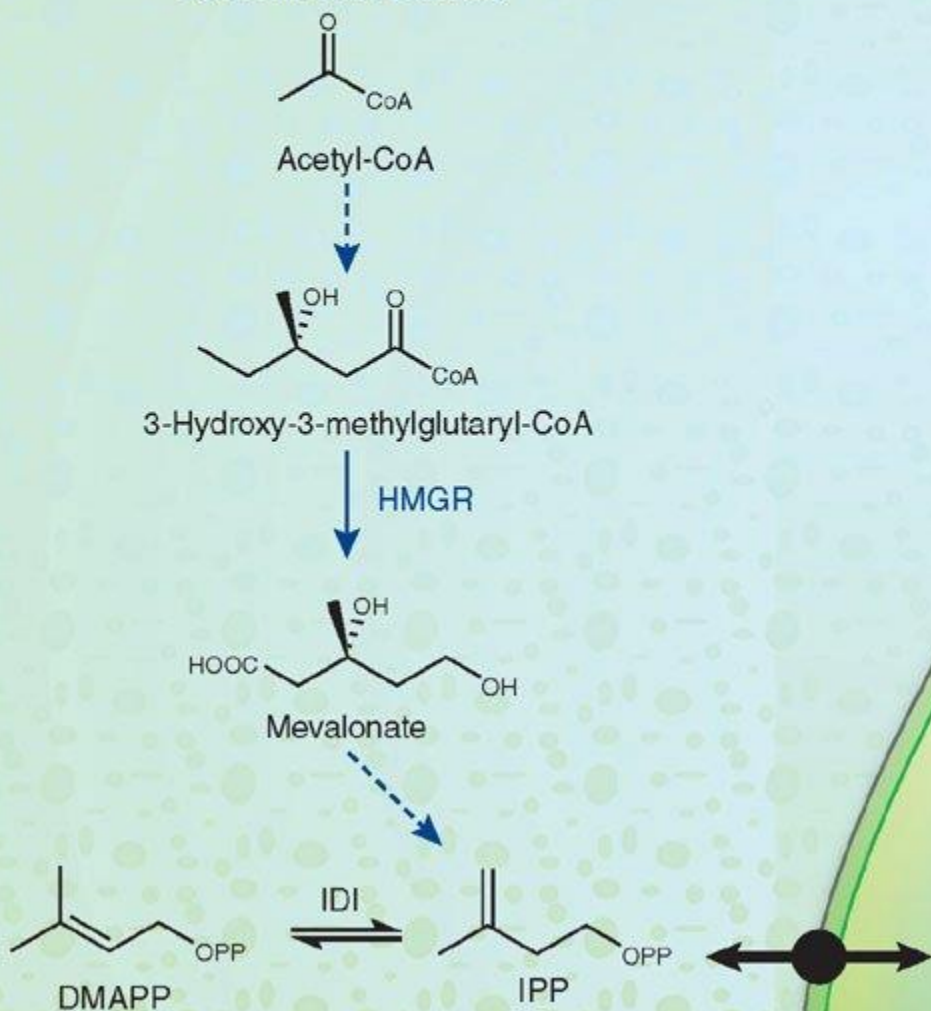
Bisabolane

2

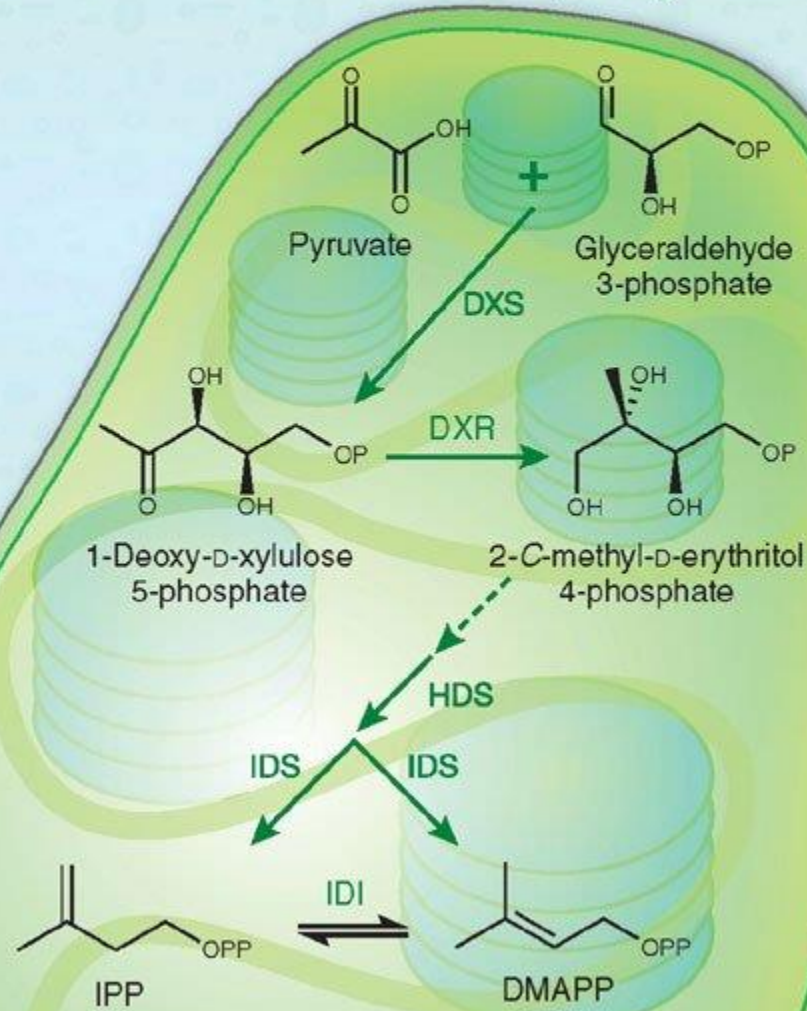
Chemical
hydrogenation

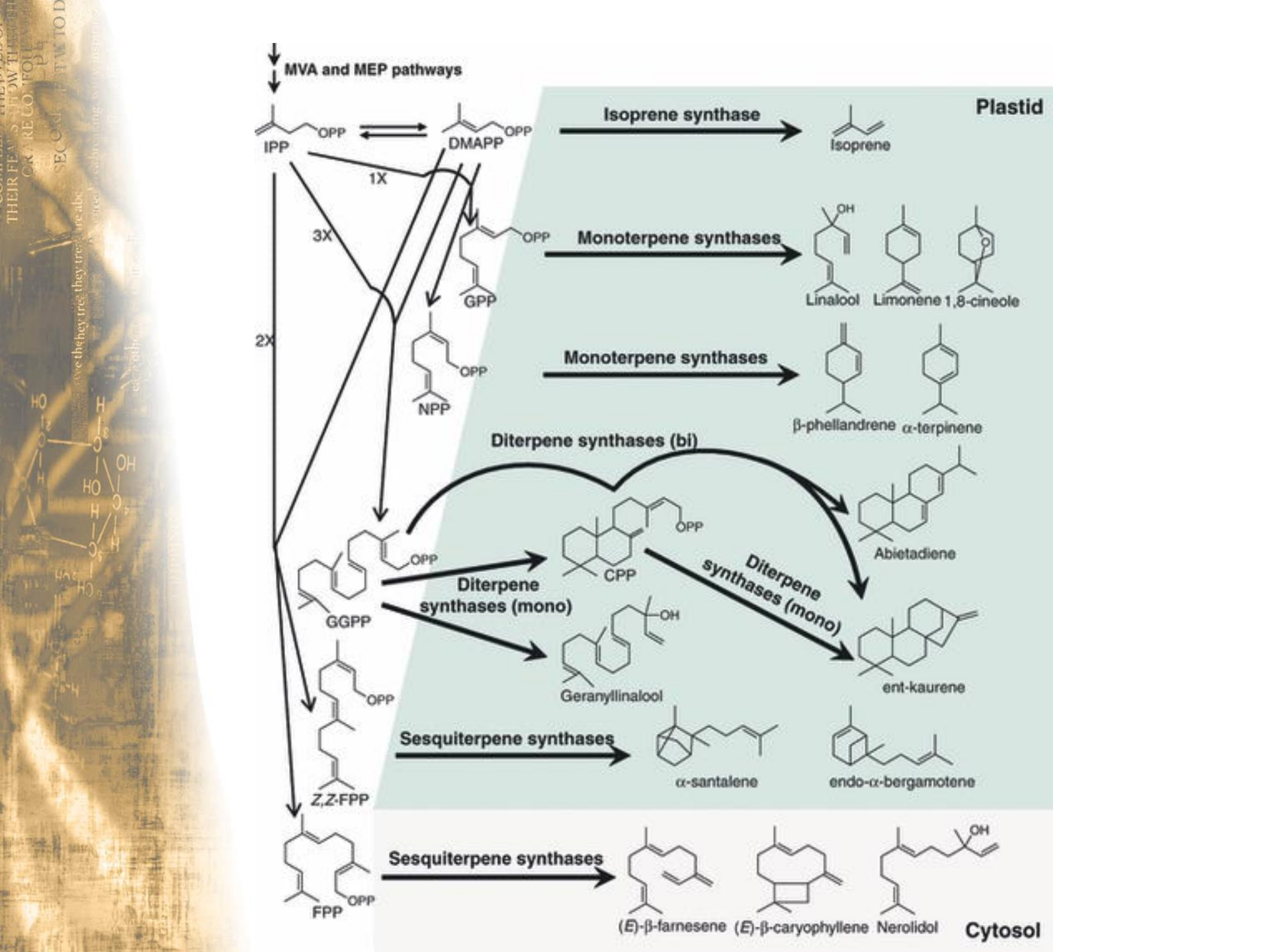


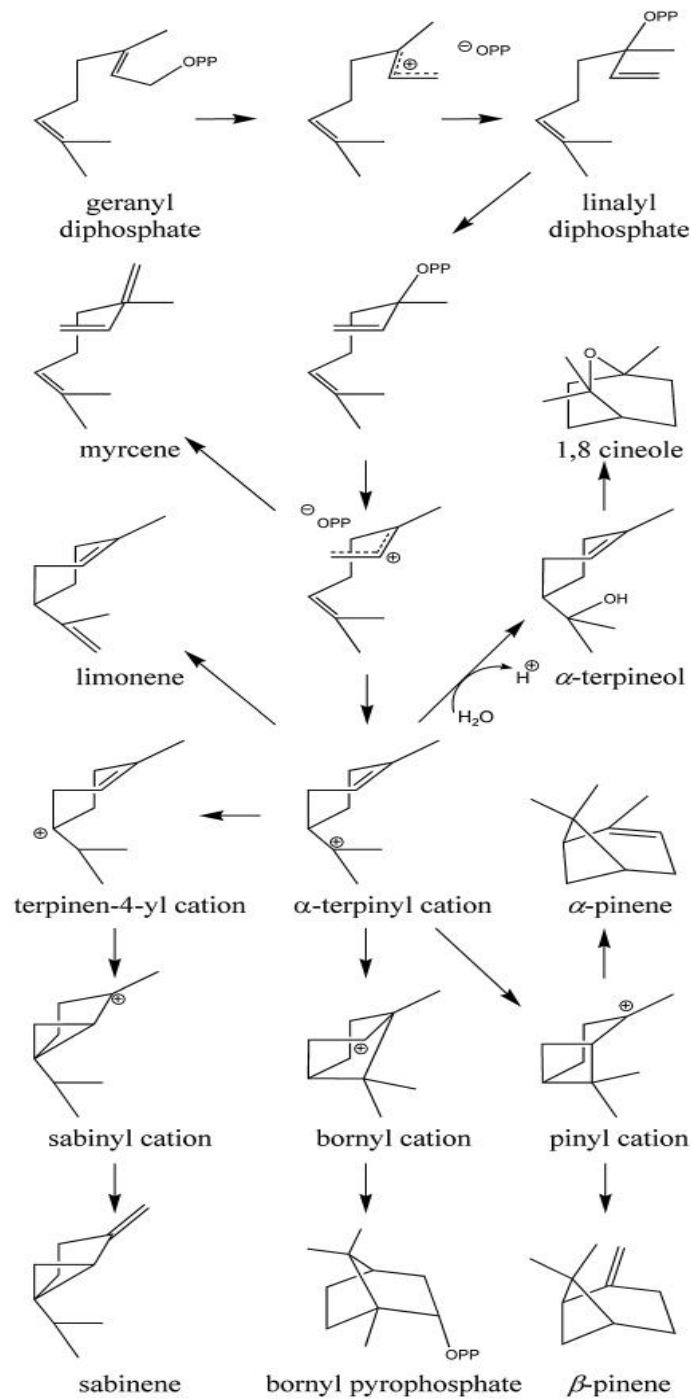
Cytosolic MVA pathway



Plastidic MEP pathway







The plants

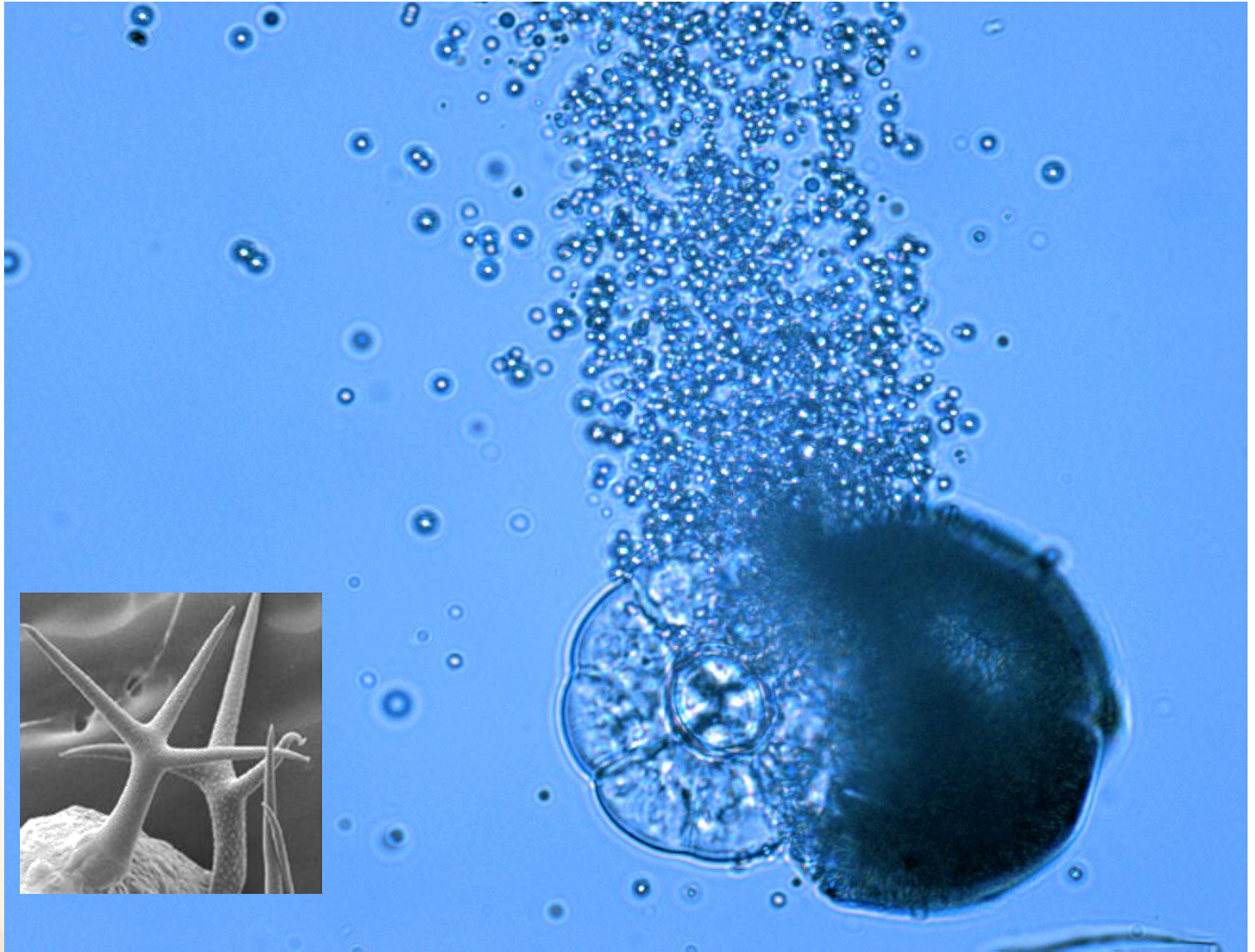
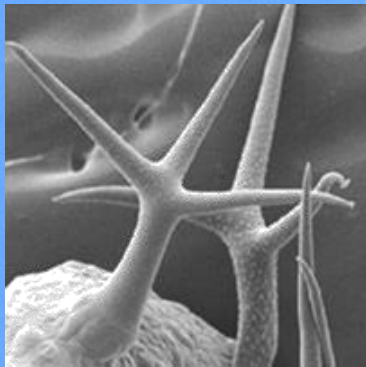
From genes to essential oils:

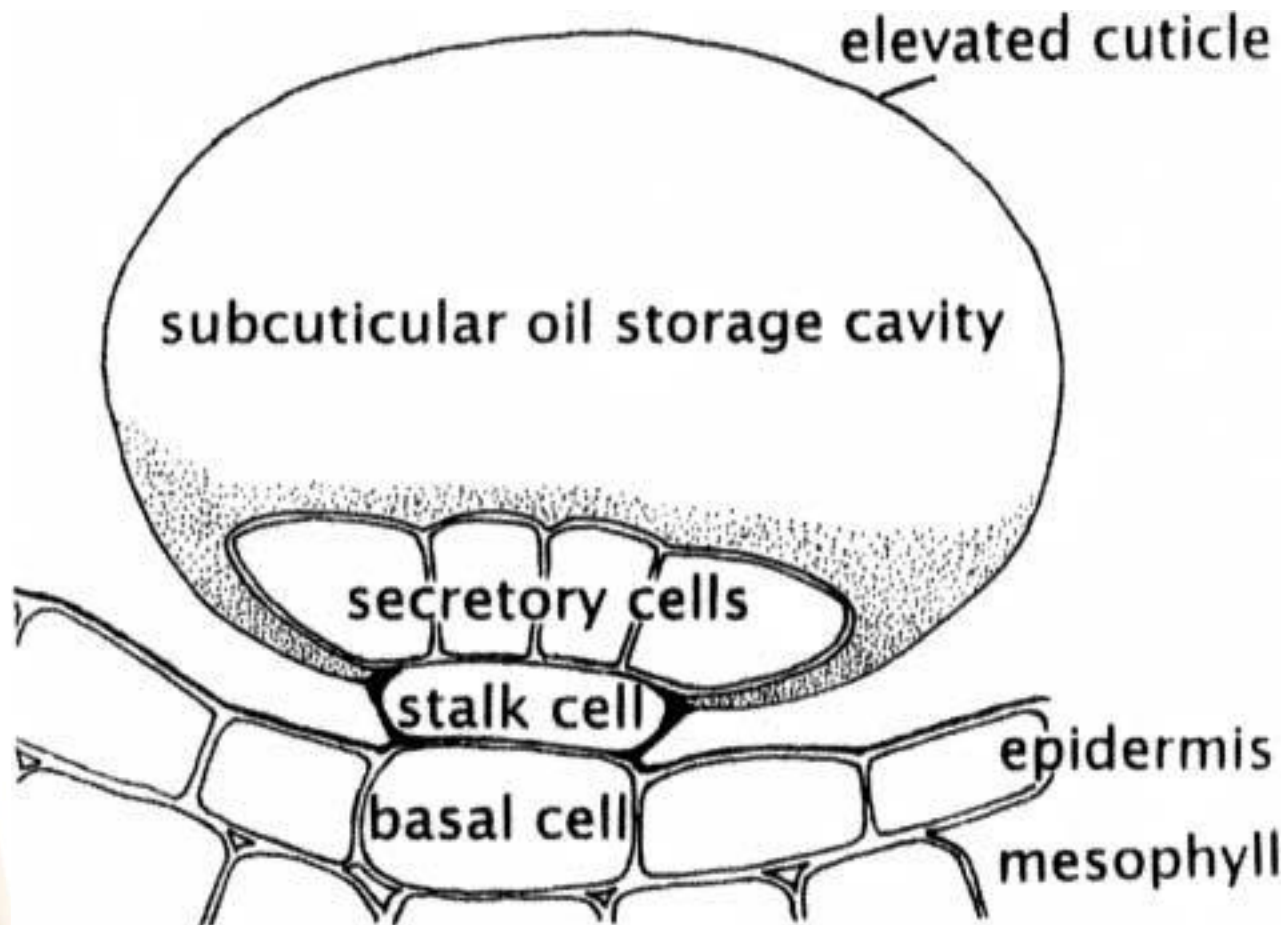
Salvia fruticosa

Mining the genes



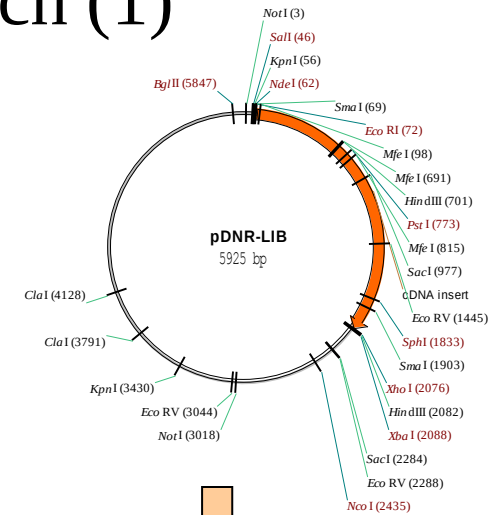
Terpenes are produced in the glandular trichomes



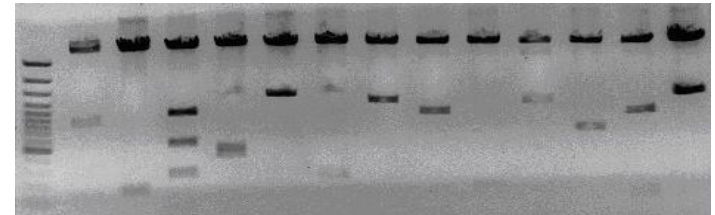


Summary of EST approach (1)

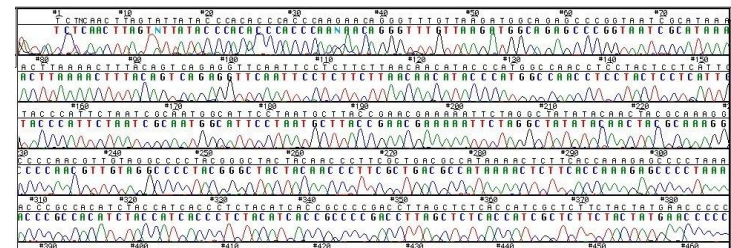
Isolation of glandular trichomes from *S. fruticosa* and *S. pomifera* by mechanical means, extraction of total RNA, and construction of cDNA library



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



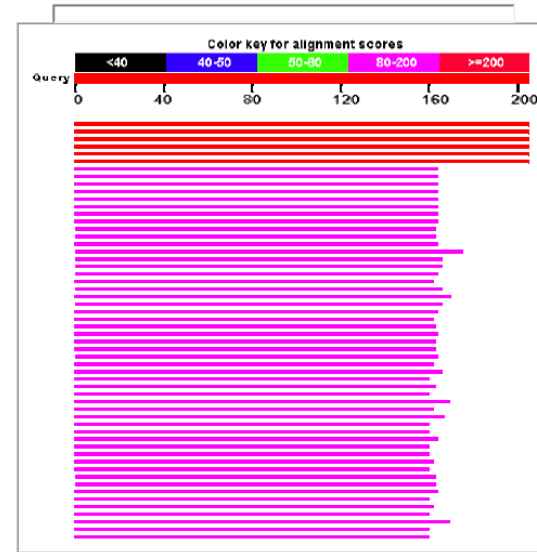
Sequencing of cDNA insert



Summary of EST approach (2)

Identification of possible gene identity by blastx

Distribution of 114 Blast Hits on the Query Sequence



Classification of isolated clones according to the MIPS functional classification of the closest *Arabidopsis* protein

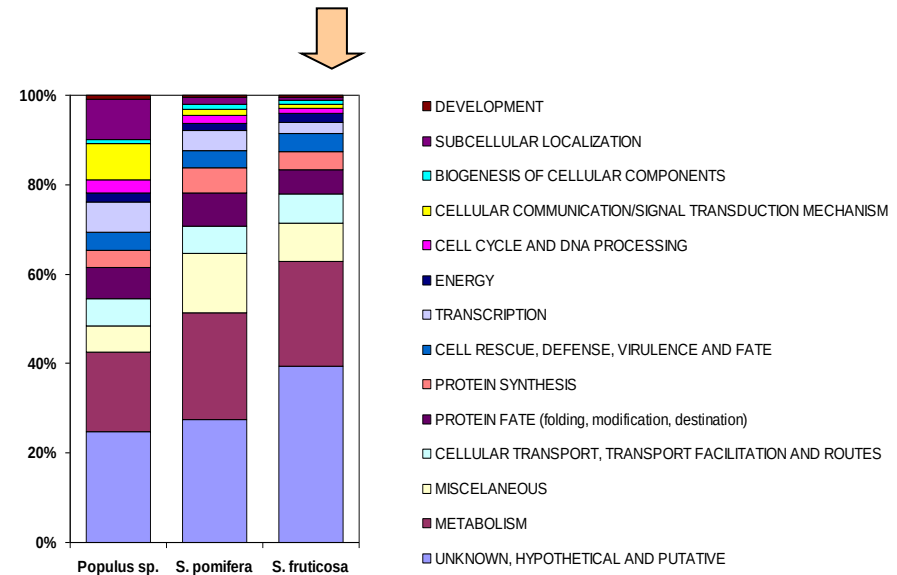


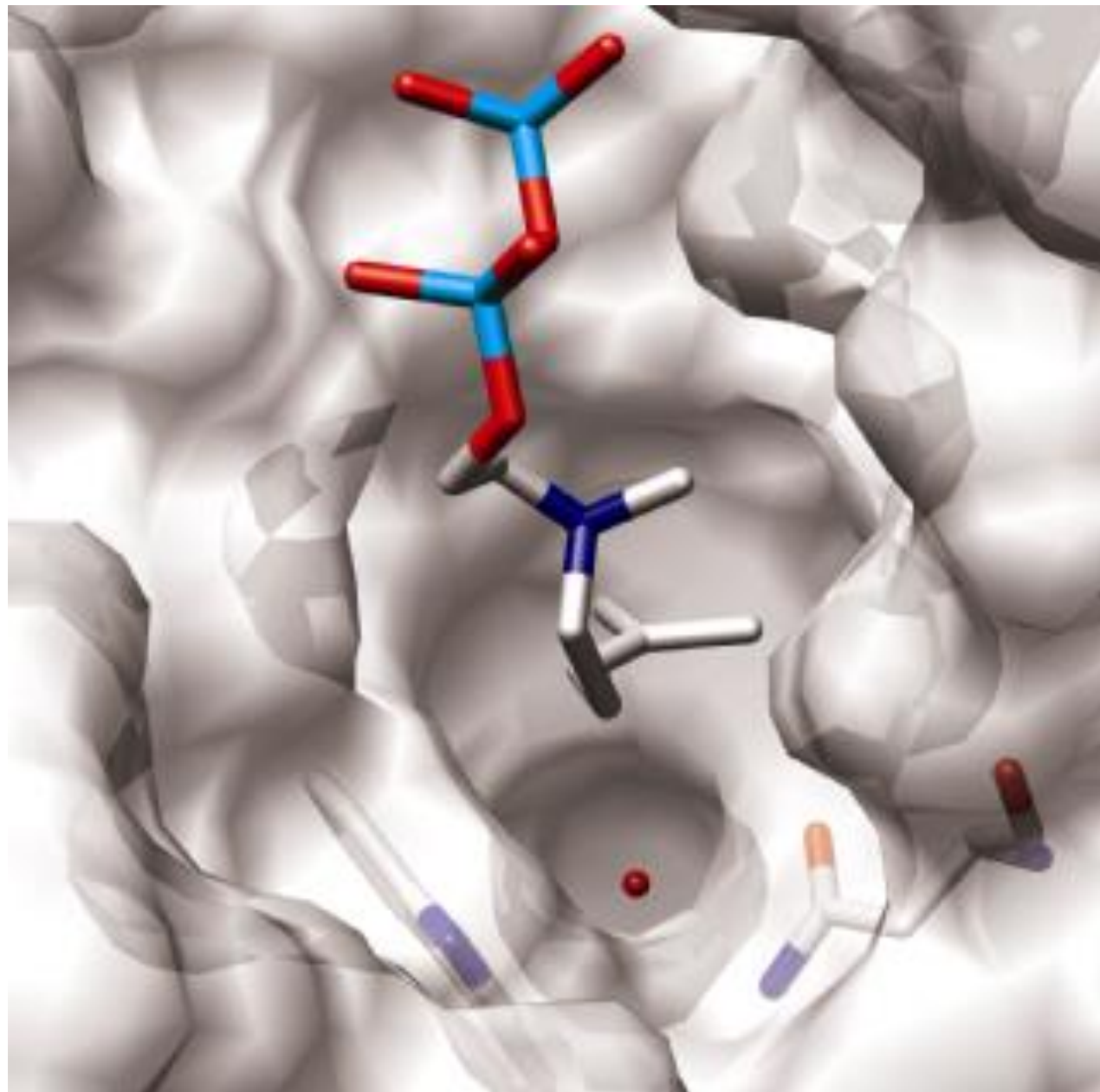
Table of Hits

<i>Function</i>	<i>S. fruticosa</i>	<i>Hits</i>	<i>S. pomifera</i>	<i>Hits</i>
Isoprene synthesis	1-deoxy-D-xylulose 5-phosphate synthase 1	1	1-deoxy-D-xylulose 5-phosphate synthase 1	1
	putative 1-deoxy-D-xylulose 5-phosphate reductoisomerase (DXR) 1 ^a	1	putative 3-Hydroxy-3-methylglutaryl-coenzyme A synthase (HMGS) 1	2
	farnesyl diphosphate synthase 1	1		
	putative 3-Hydroxy-3-methylglutaryl-coenzyme A synthase (HMGS) 1	1		
	putative 3-Hydroxy-3-methylglutaryl-coenzyme A synthase (HMGS) 2	1		
	putative xylose isomerase	1		
Monoterpene synthases	<i>1,8 cineole synthase 1</i>	5	<i>sabinene synthase 1</i>	1
	putative 1,8 cineole synthase 2	1		
	<i>putative monoterpene synthase 1</i>	1		
	truncated monoterpene synthase 1	1		
Sesquiterpene synthases	putative sesquiterpene synthase 1	2	putative sesquiterpene synthase 1	5
	putative sesquiterpene synthase 2	1	putative sesquiterpene synthase 2	1
	putative sesquiterpene synthase 3	1	putative sesquiterpene synthase 3	2
	putative sesquiterpene synthase 4	1	truncated sesquiterpene synthase 1	1
Diterpene synthases	putative copalyl diphosphate synthase 1	1	putative copalyl diphosphate synthase 1	2
			putative ent-kaurene synthase 1	1
Phenolic biosynthesis	<i>phenyl coumaran benzyl ether reductase 1</i>	7	putative caffeic acid-O-methyltransferase	1
	phenyl coumaran benzyl ether reductase 2	1	putative cinnamyl-alcohol dehydrogenase	1
	phenyl coumaran benzyl ether reductase 3	1	putative chalcone isomerase 1	1
	phenyl coumaran benzyl ether reductase 4	1	putative chalcone isomerase 2	1
	phenyl coumaran benzyl ether reductase 5	1		
	phenyl coumaran benzyl ether reductase 6	1		
	phenyl coumaran benzyl ether reductase 7	1		
Cytochrome P450s			cytochrome P450 family protein 1	1
			cytochrome P450 family protein 2	1
			cytochrome P450 family protein 3	1
			cytochrome P450 family protein 4	1
			cytochrome P450 family protein 5	1
			cytochrome P450 family protein 6	1
			cytochrome P450 family protein 7	1
			cytochrome P450 family protein 8	1
			cytochrome P450 family protein 9	1
			cytochrome P450 family protein 10	1
			cytochrome P450 family protein 11	1
			cytochrome P450 family protein 12	1

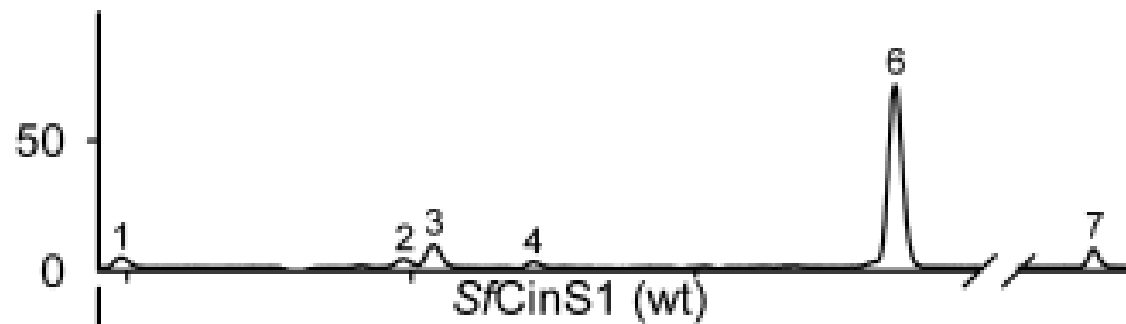
^aPutative is below 90% identity with a characterised protein.

THEY FEEL A SLOW TREMOR
OR A RECOIL FROM THE
SECOND PLATE TO D
...the they try, they try, are ab
...ing, who consp
...the they try, they try, are ab
...ing, who consp
...the they try, they try, are ab
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The cineole
synthase
active site
region
showing the
water
molecule
associated
with
Asn 338



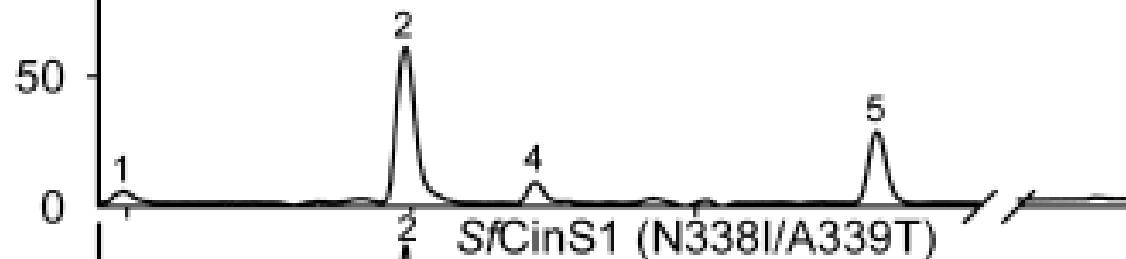
**Sf Cineole
synthase**



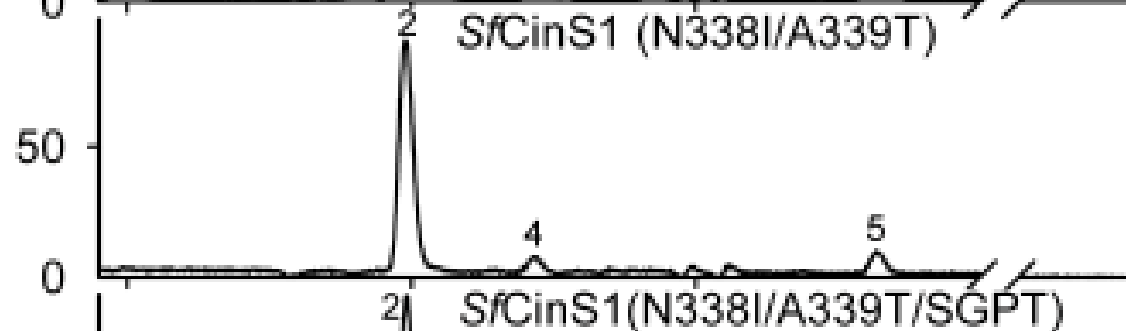
Asn338 > Ile



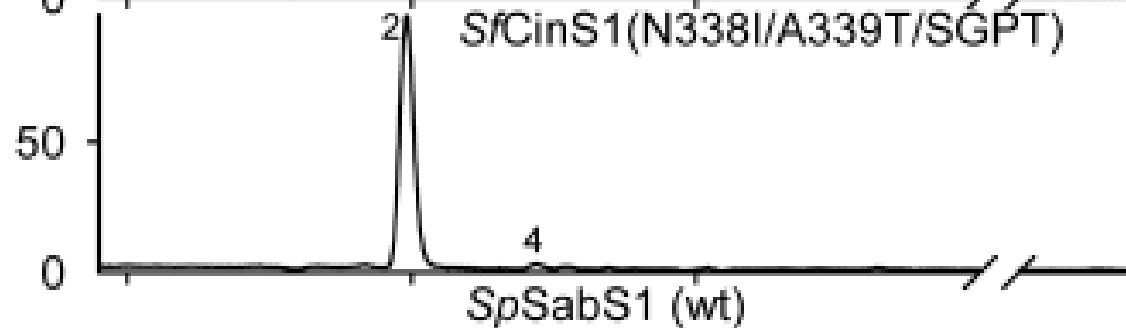
**Asn338 > Ile
Ala339 > Thr**



**Asn338 > Ile
Ala339 > Thr
Move Pro**

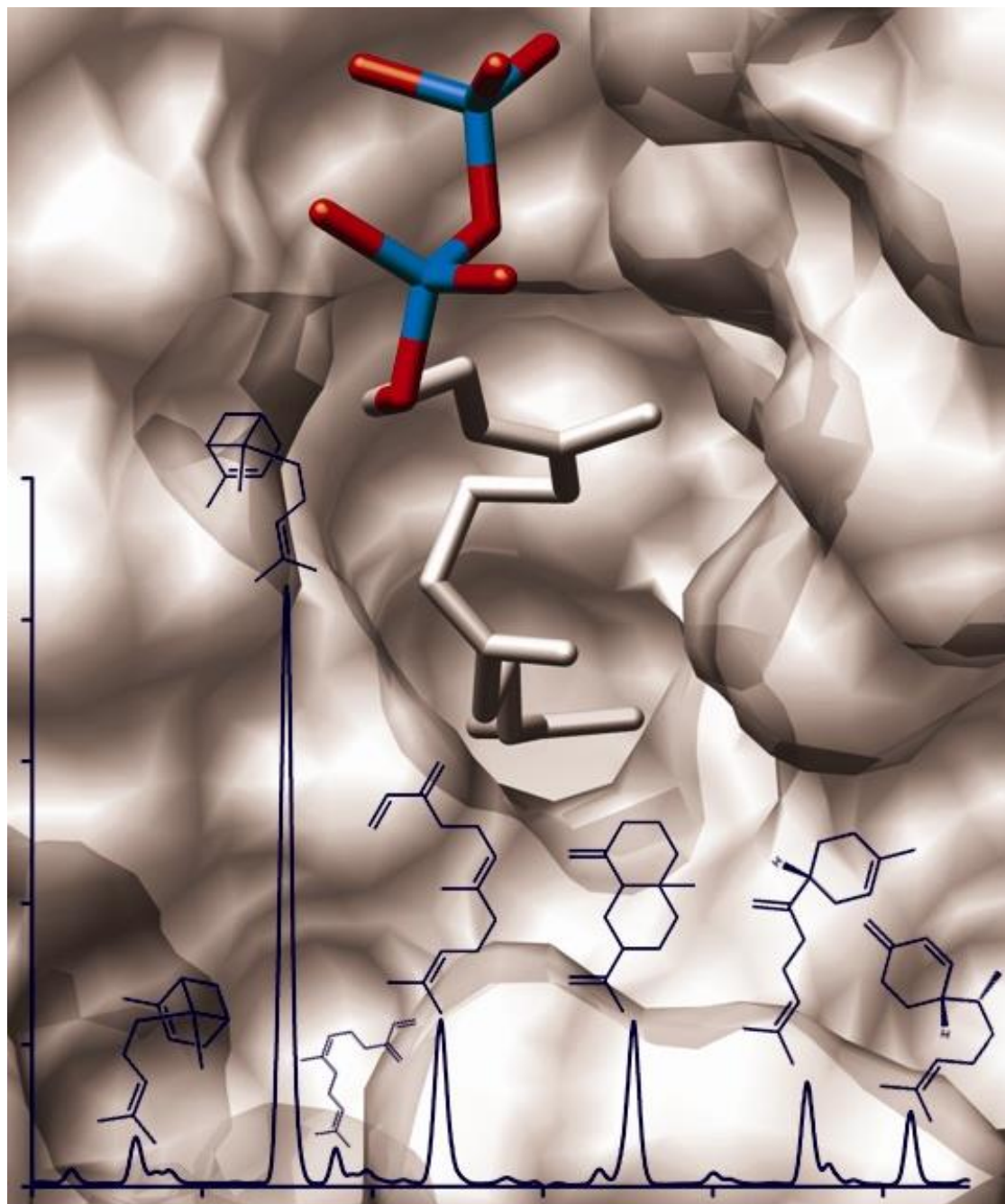


**Sp sabinene
synthase**



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?



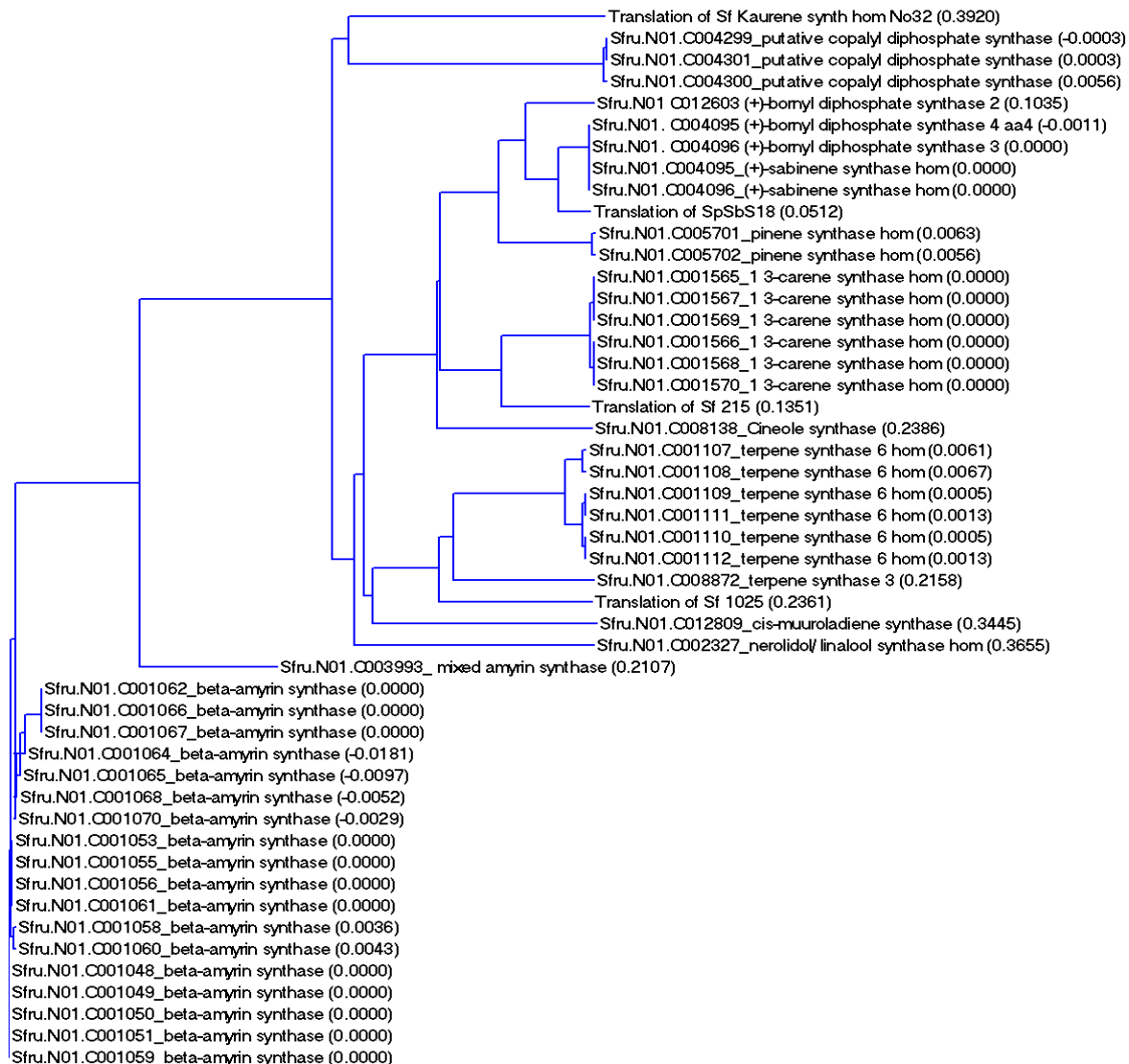
Sesquiterpene synthase activities resulting from one amino acid change (Asn338)

Enzyme variant	<i>cis</i> - α - bergamotene	<i>trans</i> - α - bergamotene	Z- β -farnesene	E- β -farnesene	β -selinene	β -bisabolene	β -sesqui phellandrene
<i>SfCinS1</i> (N338A)	4.0	49.0	2.6	16.8	15.1	9.2	6.0
<i>SfCinS1</i> (N338S)	0.0	0.0	0.0	39.4	0.0	45.1	15.4
<i>SfCinS1</i> (N338C)	5.9	45.8	3.1	17.7	13.5	10.0	7.0

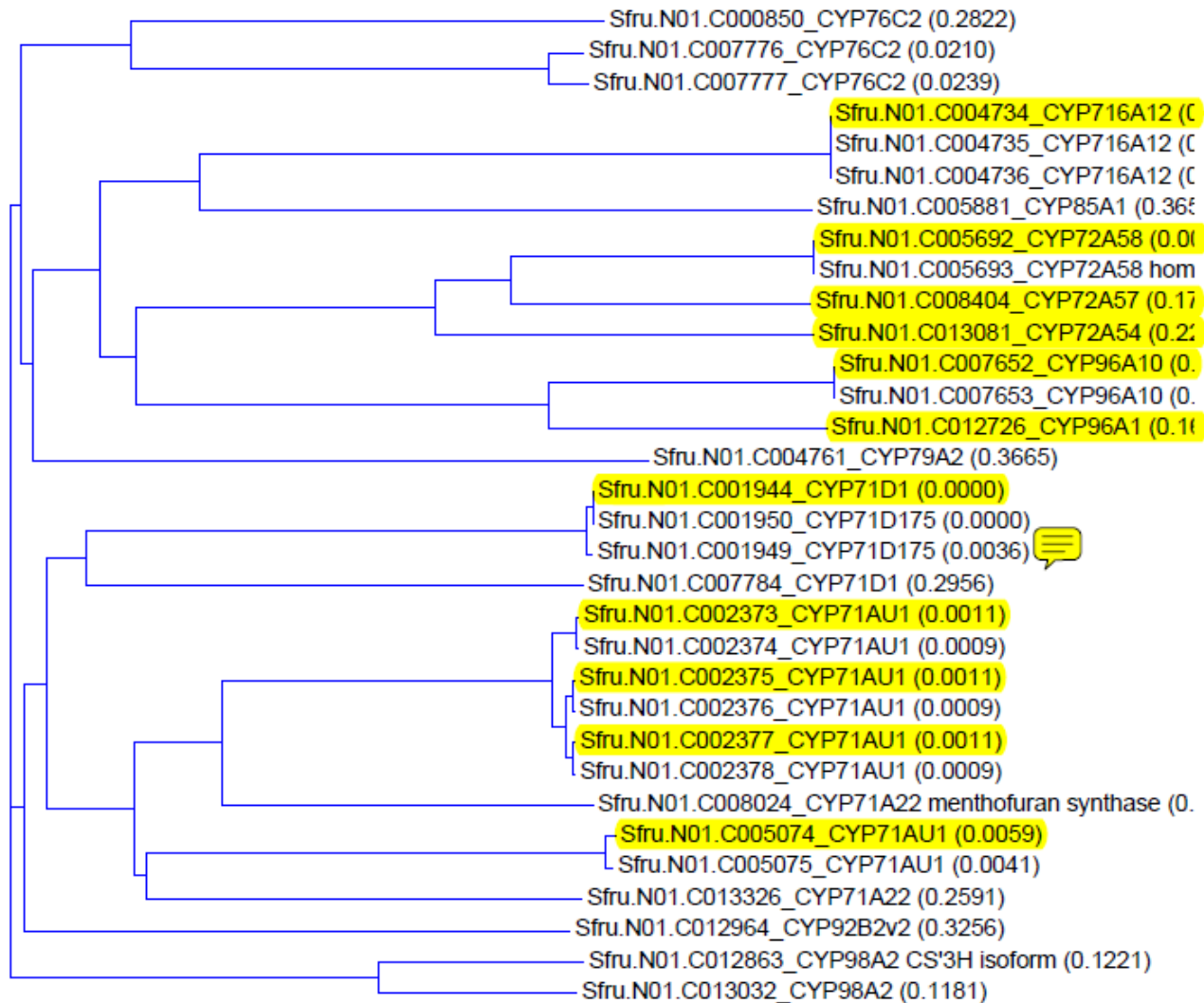
Analysis of *S. fruticosa* transcriptome data

- 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



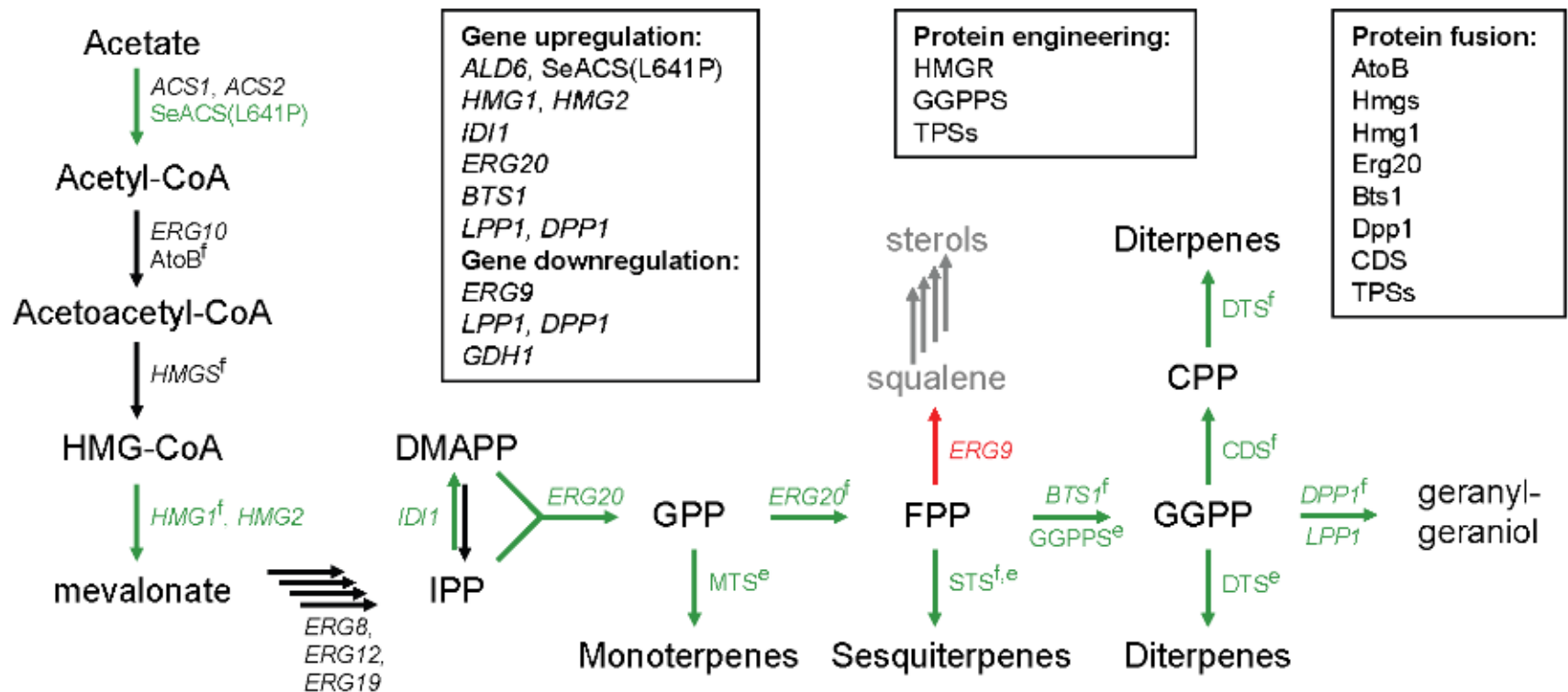
Selected genes
In yellow

“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



(^e) 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

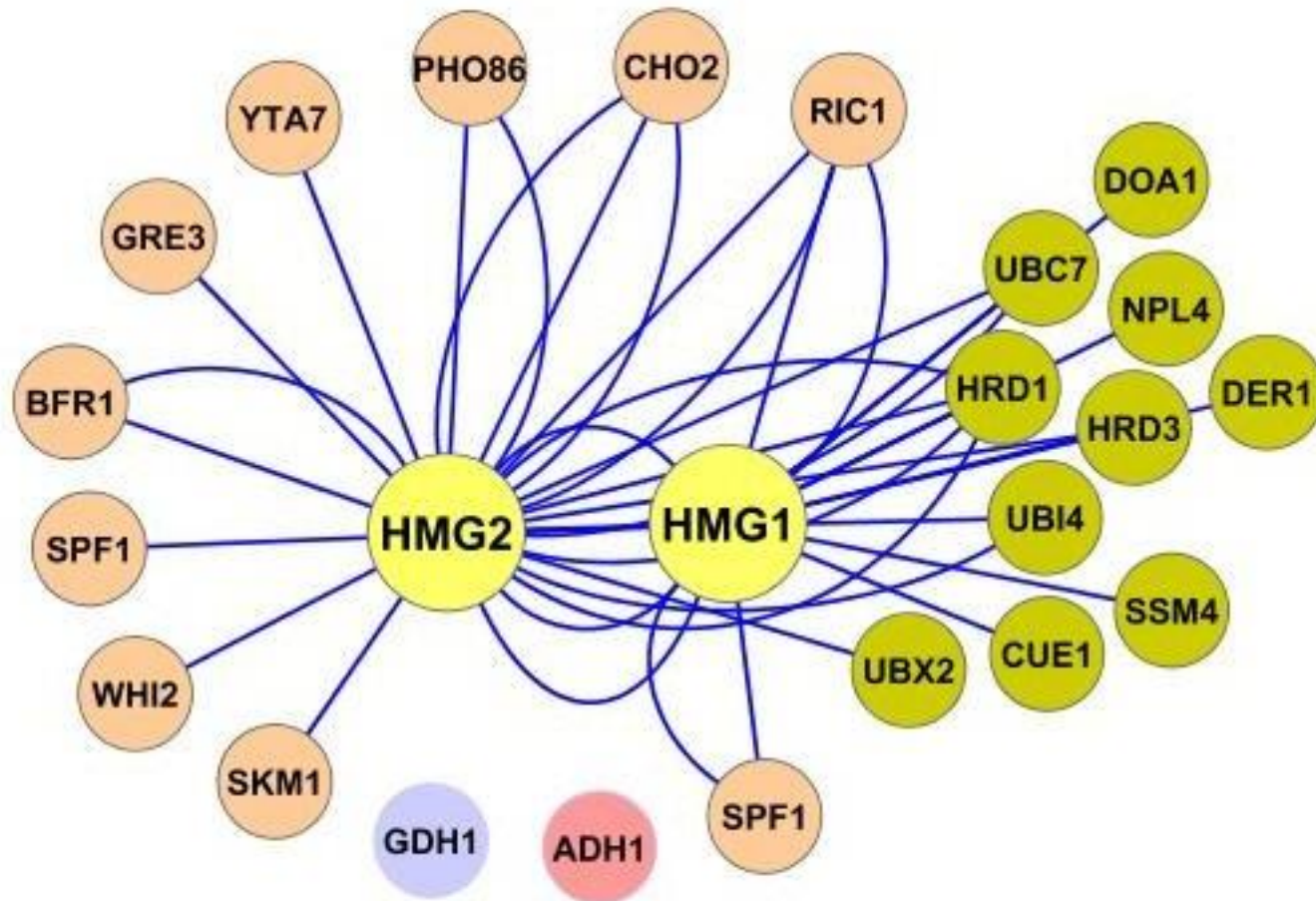
Genetic interaction in yeast

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

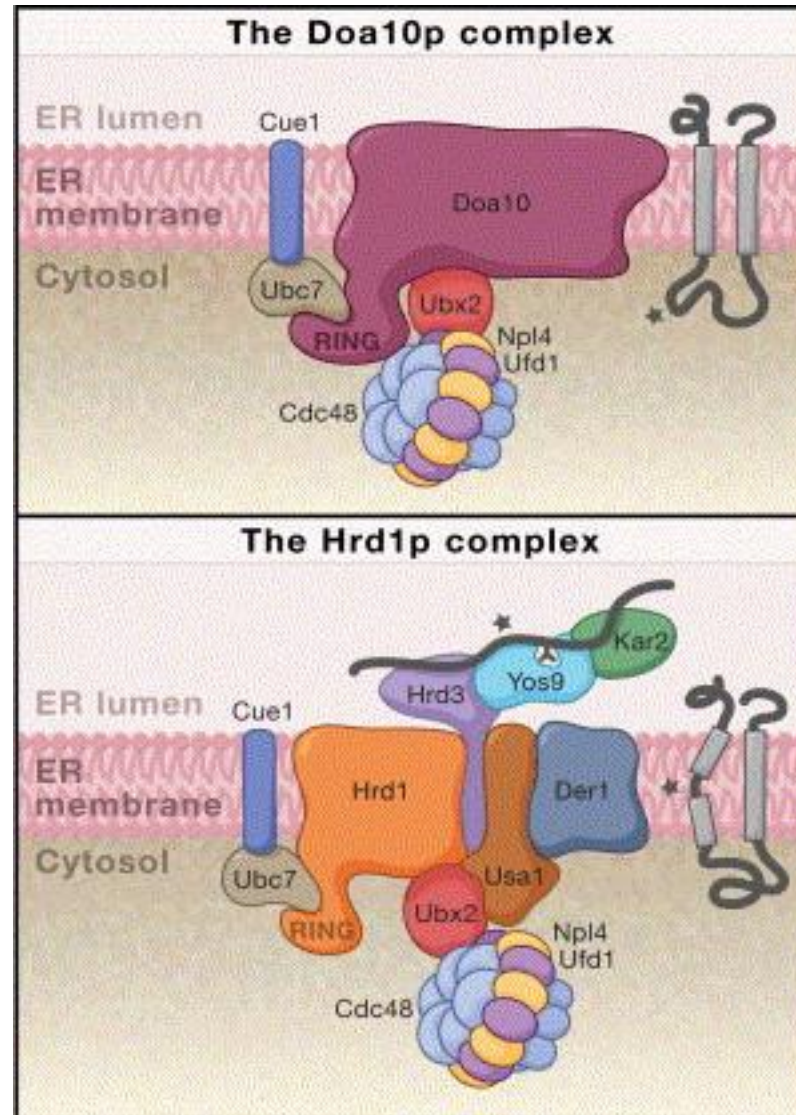
Negative interactions refer to a more severe fitness defect than expected, with the extreme case being synthetic lethality

Positive interactions refer to double mutants with a less severe fitness defect than expected

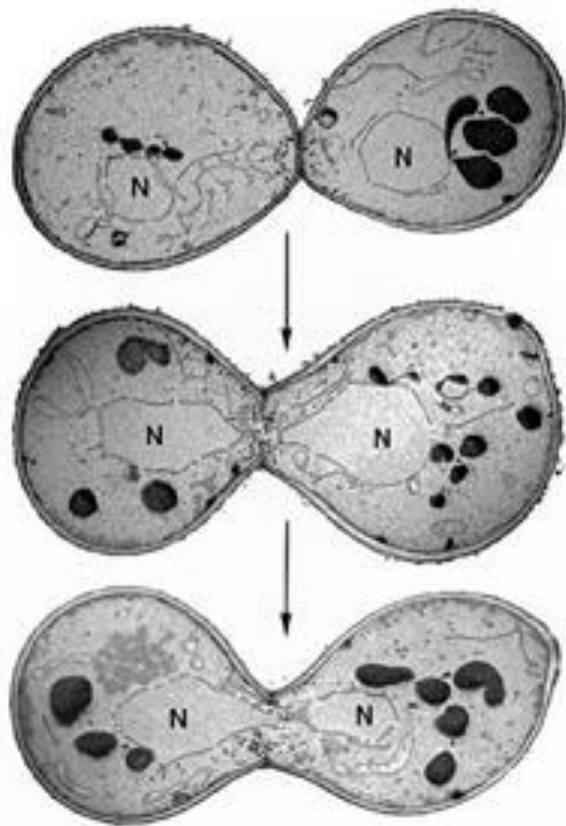
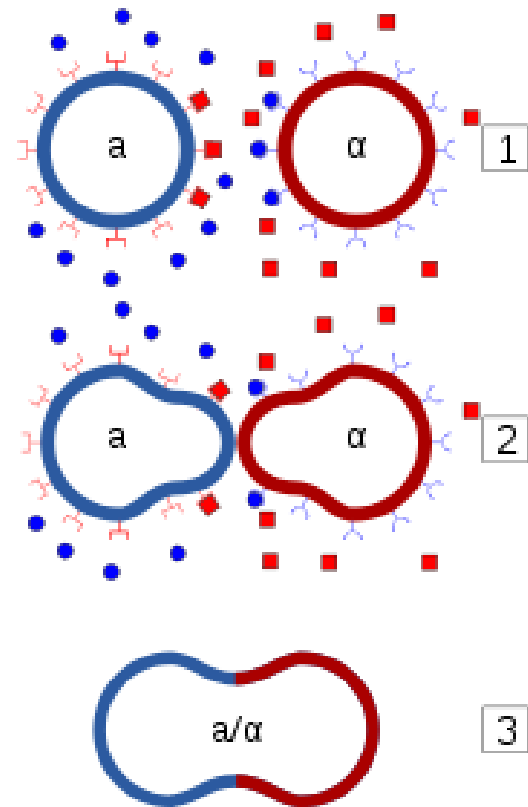
Positive Genetic interactors in yeast identify a new set of targets for improvement

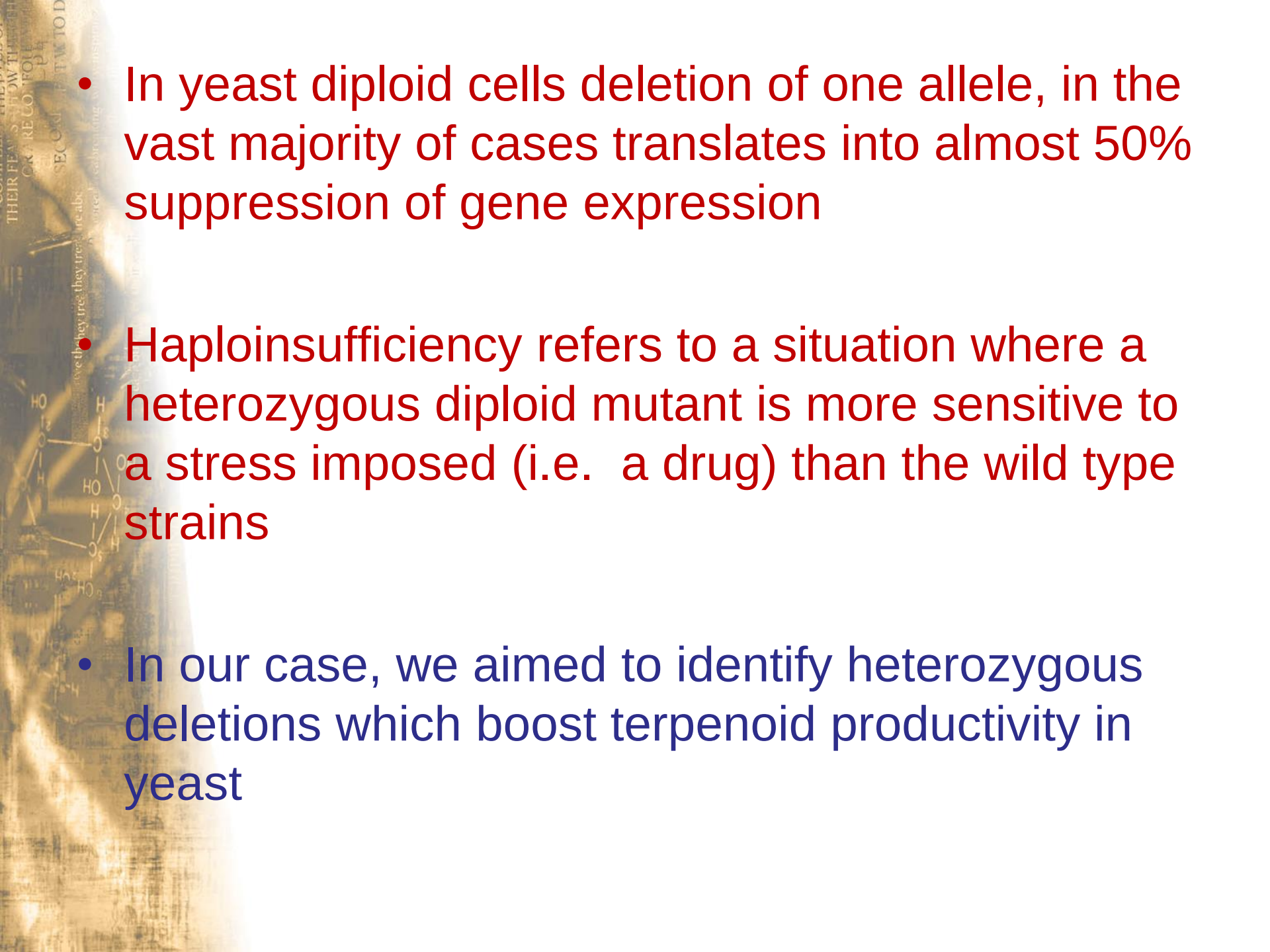


The ERAD complex



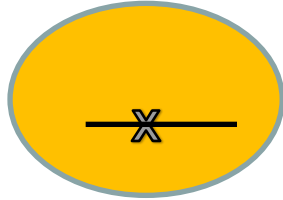
Yeasts do it ..



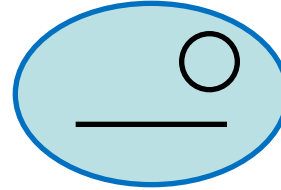
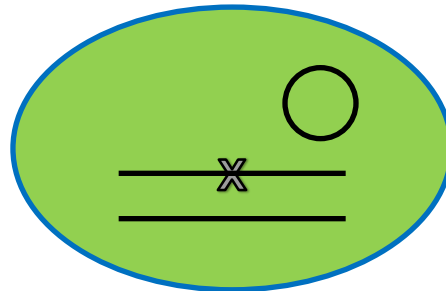
- 
- In yeast diploid cells deletion of one allele, in the vast majority of cases translates into almost 50% suppression of gene expression
  - Haploinsufficiency refers to a situation where a heterozygous diploid mutant is more sensitive to a stress imposed (i.e. a drug) than the wild type strains
  - In our case, we aimed to identify heterozygous deletions which boost terpenoid productivity in yeast

- Developing heterozygous deletion strains

Mat a deletion mutant

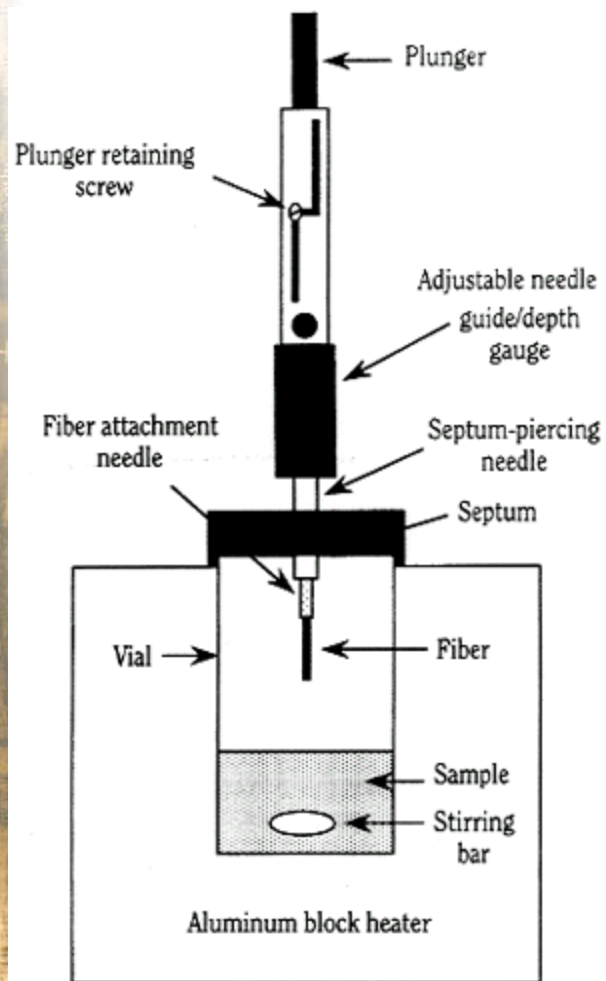
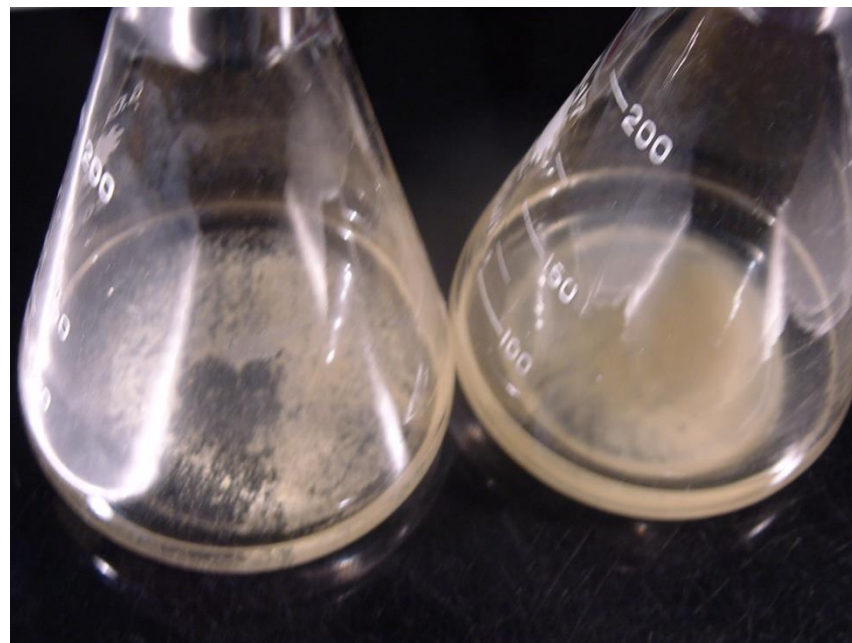


Mat alpha,
pUTDH3m/Sf126 ca

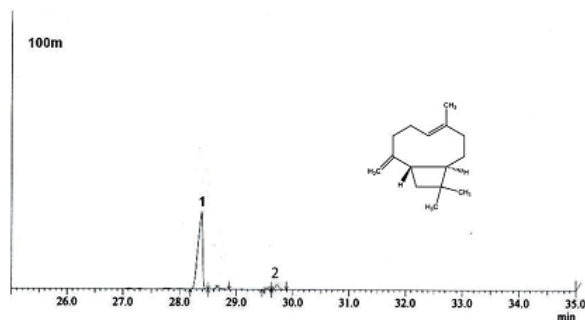


Mat a/alpha, x/X, pUTDH3m/SF126

Solid Phase Microextraction (SPME) sampling procedure

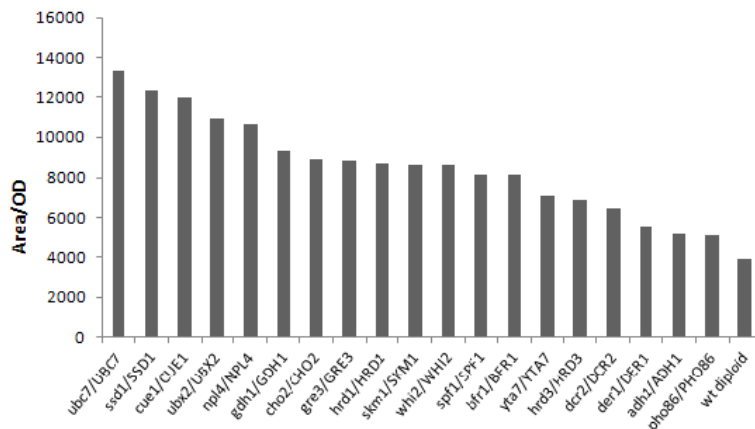


A



- Screening for heterozygous deletions which enhance terpenoid productivity

B

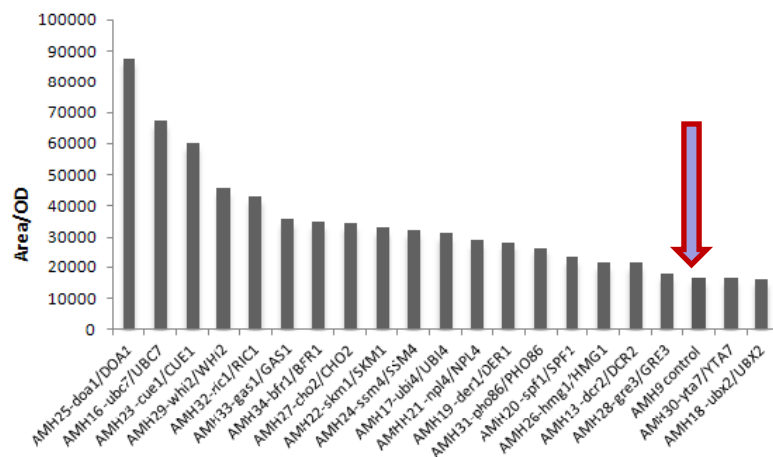


WT Diploid

Gen. background

Mat a/alpha, x/X, pUTDH3m/Sf126

C

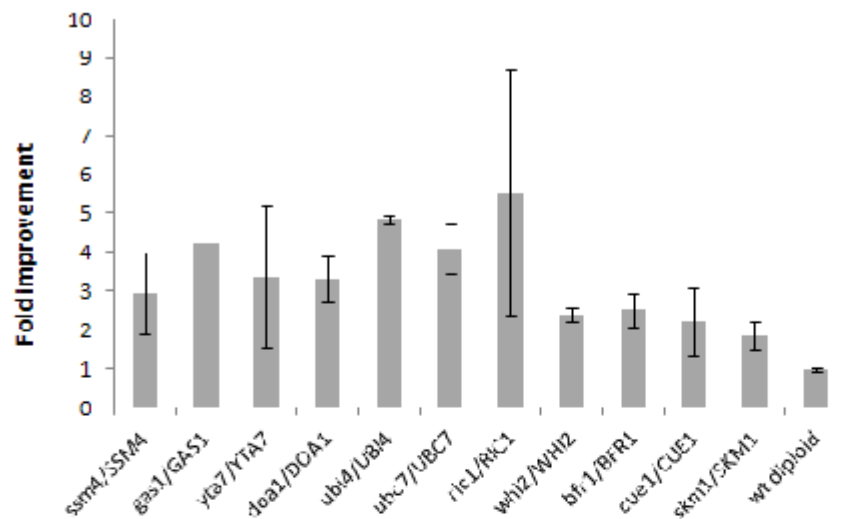


Gen. background

Mat a/alpha, P_{TDH3}-HMG2 (K6R)::HO1, pUTDH3m/Sf126

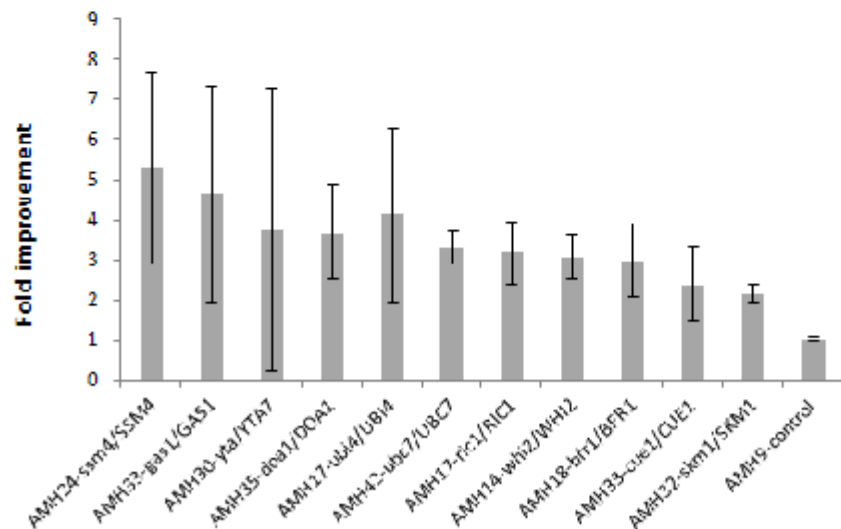
THEIR FEARS SHOW THEM
OR ARE CO-FOUNDED
SECOND FEAR TO
live the heaviest they trees are abo

A

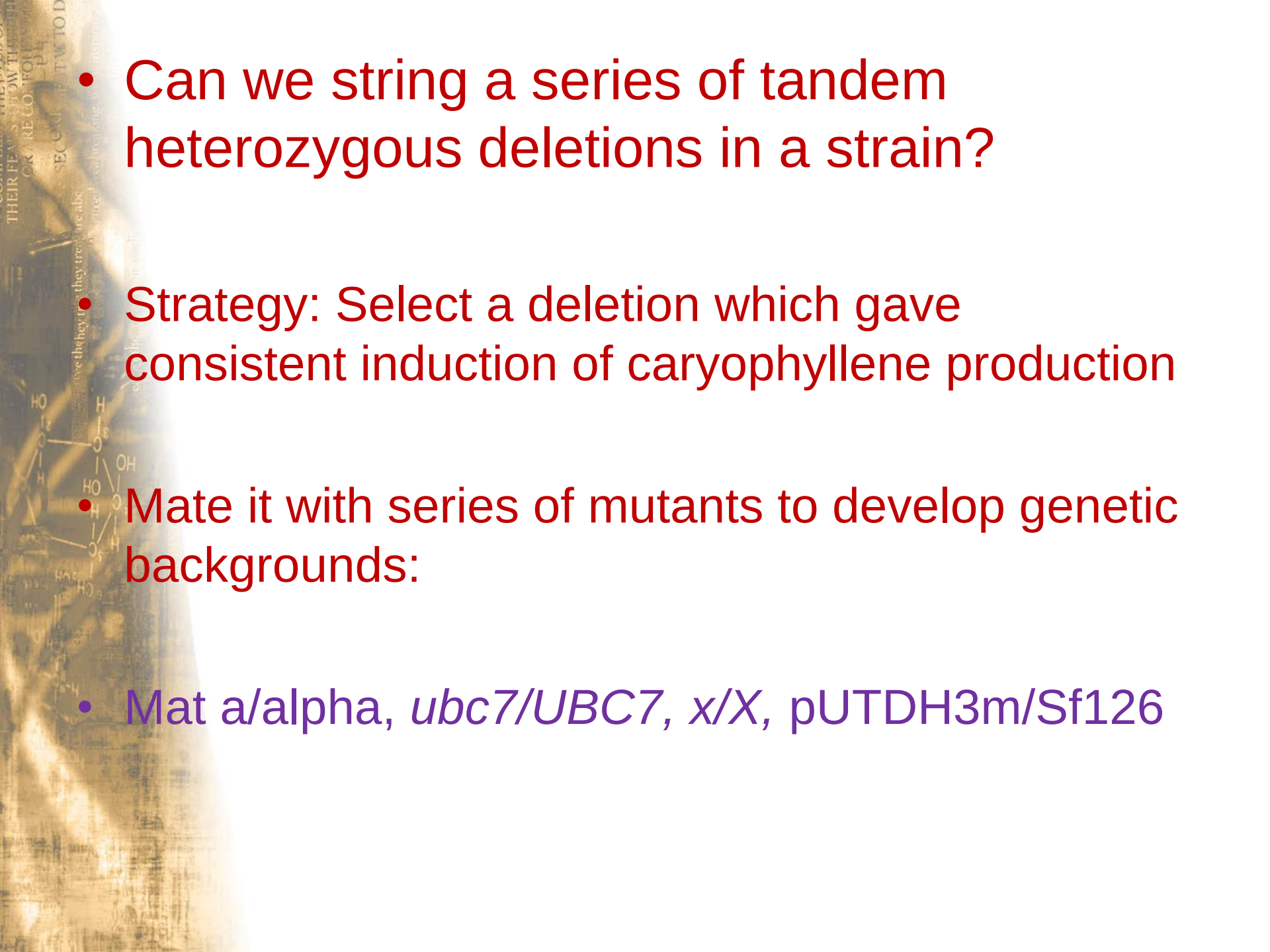


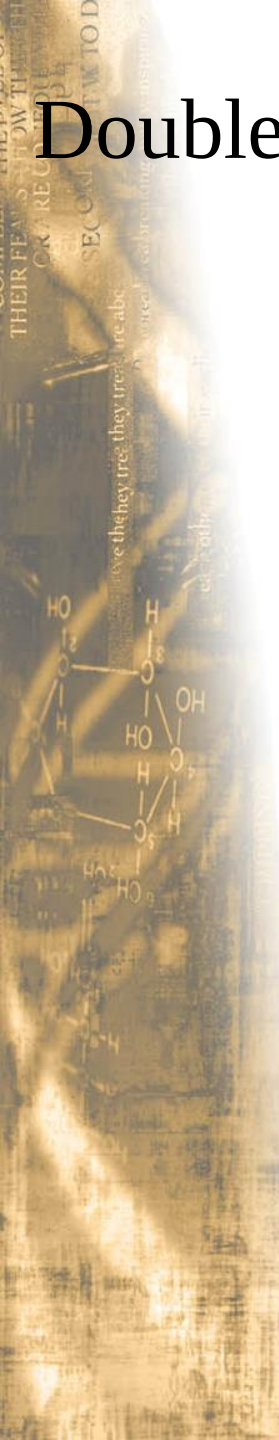
Heterozygous deletions reproducibly increase caryophyllene yields over time

B



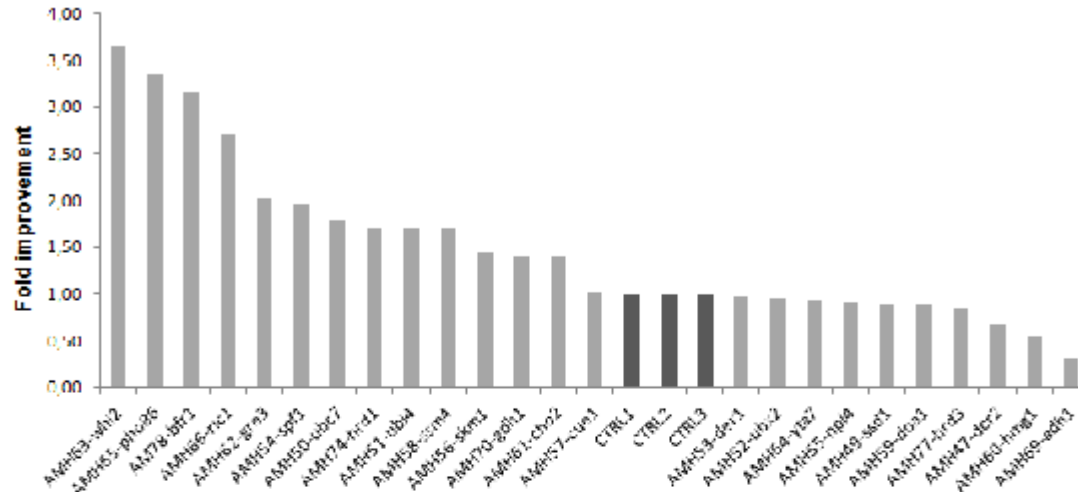
2-5 fold induction over control strain

- 
- 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 α/α , *ubc7/UBC7*, *x/X*, pUTDH3m/Sf126

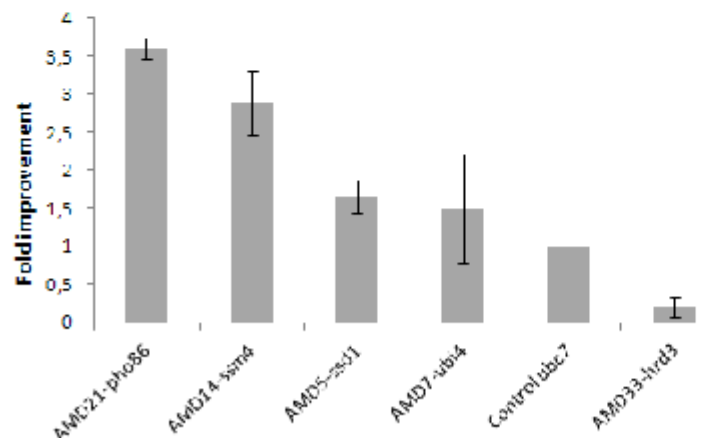


Double heterozygous and homozygous deletions in *ubc7*

A



B

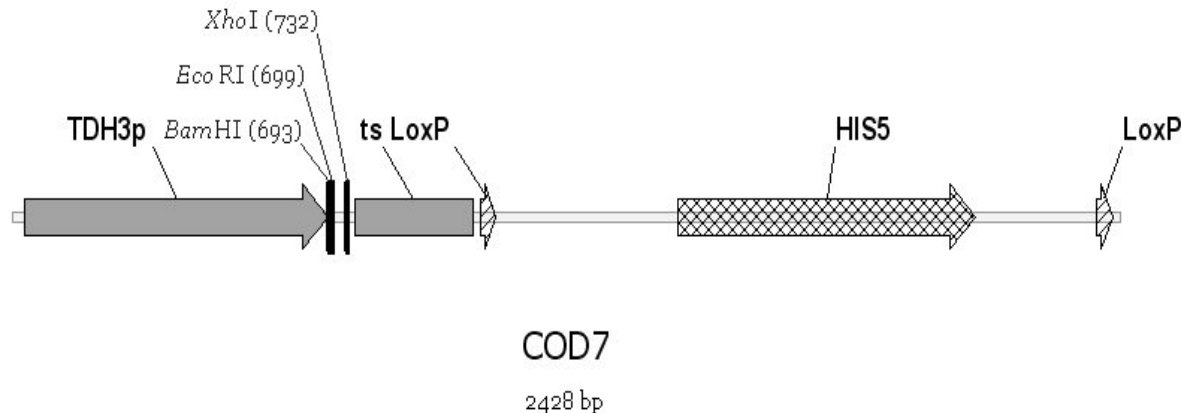


In heterozygous strains, tandem deletions lead in many cases to additional yield increases

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

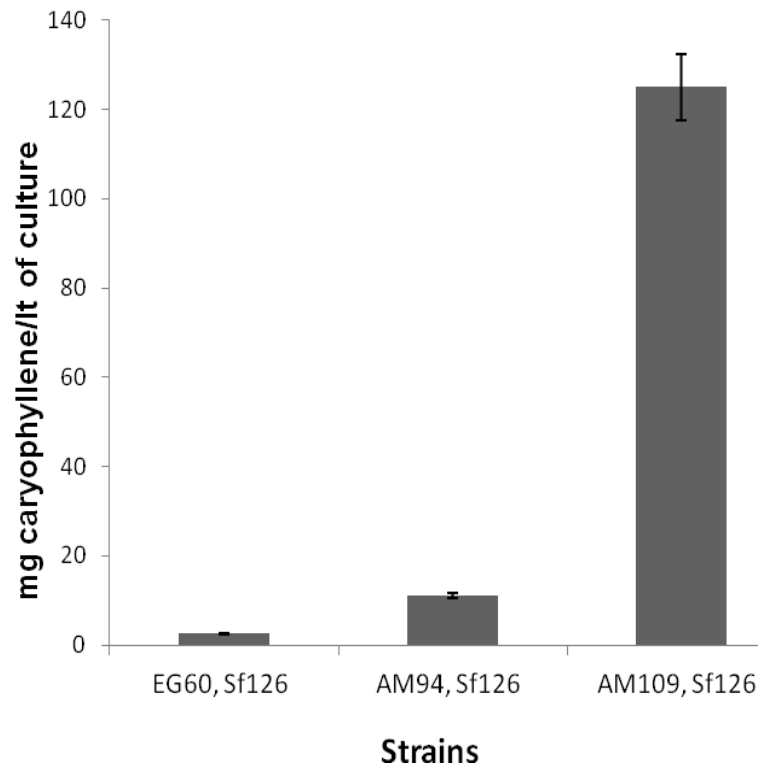
Selected genes: UBC7, SSM4/DOA10, PHO86

Engineered new yeast strains for high caryophyllene production

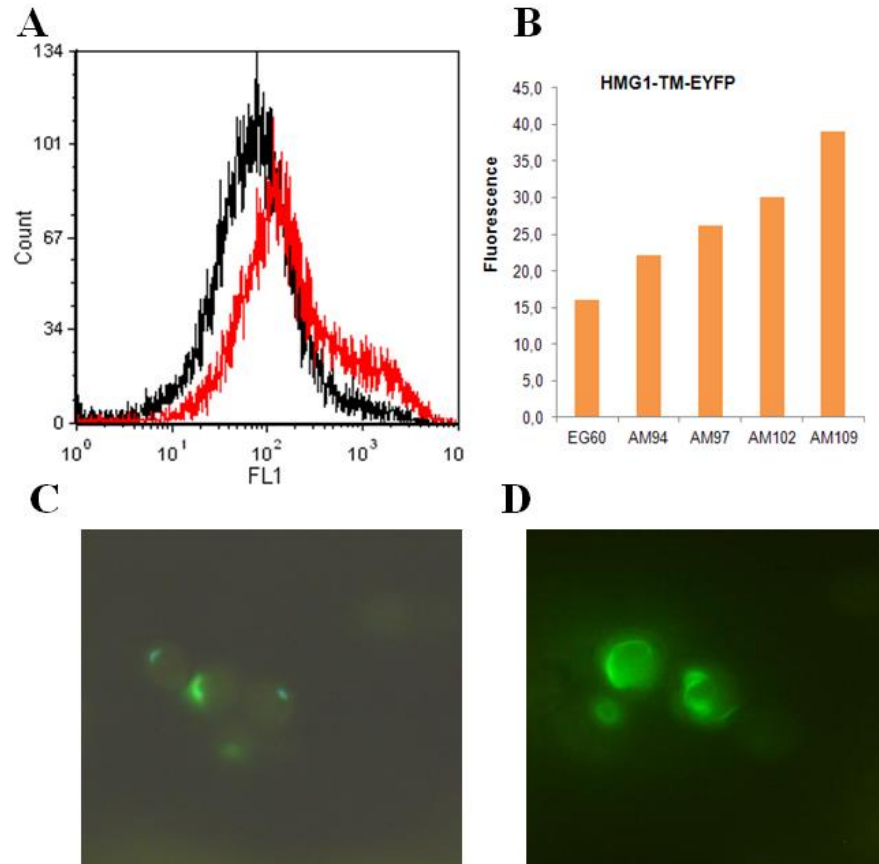


EG60-01	Mata α , <i>ura3 trp1 his3</i>	pUTDH3/Sfl26, 2 μ URA3 P _{TDH3} -Sfl 26
AM94-01	Mata α / α , P _{GAD} -HMG2(K6R)::HOX2, <i>ura3 trp1 his3</i> , P _{TDH3} -HMG2(K6R):: <i>leu2 X2, ERG9/erg9</i> Derived from AM90	pUTDH3/Sfl26, 2 μ URA3 P _{TDH3} -Sfl 26
AM97-01	Mata α / α , P _{GAD} -HMG2(K6R)::HOX2, <i>ura3 trp1 his3</i> , P _{TDH3} -HMG2(K6R)X2:: <i>leu2 ERG9/erg9, UBC7/hbc7</i> derived from AM94	pUTDH3/Sfl26, 2 μ URA3 P _{TDH3} -Sfl 26
AM102-01	Mata α / α , P _{GAD} -HMG2(K6R)::HOX2, <i>ura3 trp1 his3</i> , P _{TDH3} -HMG2(K6R):: <i>leu2 X2, ERG9/erg9, UBC7/hbc7, SSM4/ssm4</i> derived from AM97	pUTDH3/Sfl26, 2 μ URA3 P _{TDH3} -Sfl 26
AM109-01	Mata α / α , P _{GAD} -HMG2(K6R) $\times$ 2::HOX2, <i>ura3 trp1 his3</i> , P _{TDH3} -HMG2(K6R)X2:: <i>leu2 ERG9/erg9, UBC7/hbc7, SSM4/ssm4, PHO86/pho86</i> derived from AM102	pUTDH3/Sfl26, 2 μ URA3 P _{TDH3} -Sfl 26

Improved strains produce 50-fold higher yields of caryophyllene in yeast



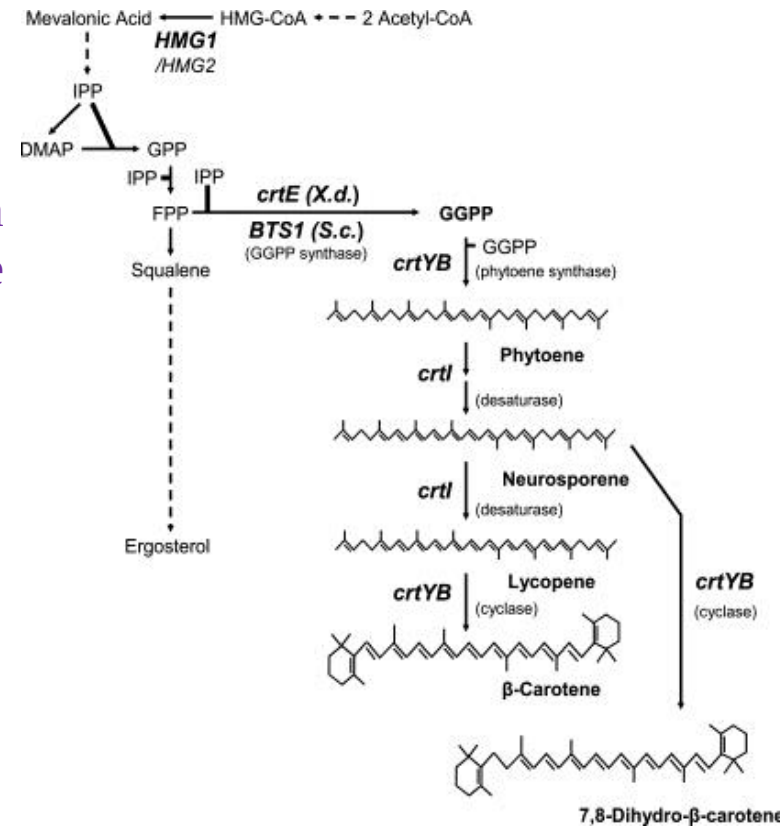
Heterozygous deletions increase Hmgp stability in engineered cells



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

APPLIED AND ENVIRONMENTAL MICROBIOLOGY, July 2007, p. 4342–4350
0099-2240/07/\$08.00+0 doi:10.1128/AEM.02759-06
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Vol. 73, No. 13

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

René Verwaal,^{1†} Jing Wang,¹ Jean-Paul Meijnen,¹ Hans Visser,^{1‡} Gerhard Sandmann,²
Johan A. van den Berg,¹ and Albert J. J. van Ooyen^{1*}

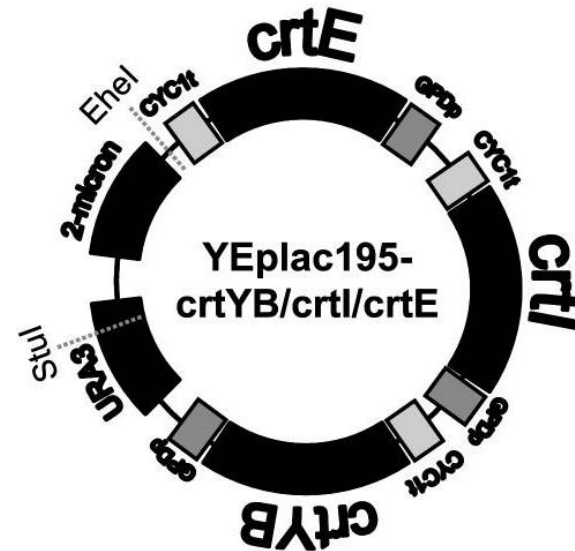
Fungal Genomics, Laboratory of Microbiology, Wageningen University, Dreijenlaan 2, 6703 HA Wageningen, The Netherlands,¹ and
Biosynthesis Group, Molecular Biosciences 213, J. W. Goethe Universität, Siesmayerstrasse 70, P.O. Box 111932,
D-60054 Frankfurt, Germany²

Received 26 November 2006/Accepted 5 May 2007

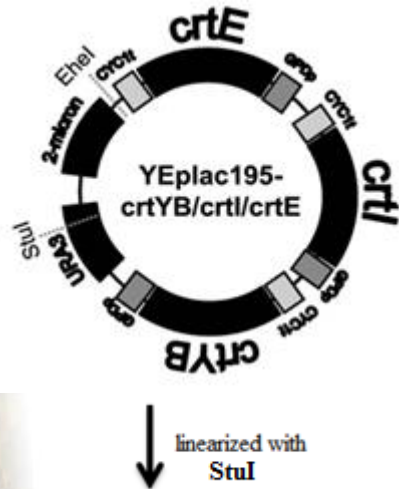
crtE : GGPP synthase

crtYB : phytoene synthase

crtI : phytoene desaturase

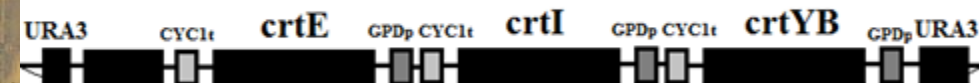
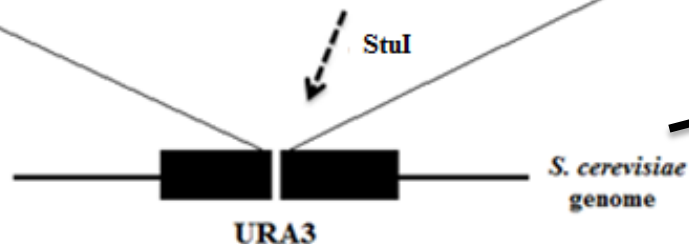


Plasmid digest and yeast strain transformation



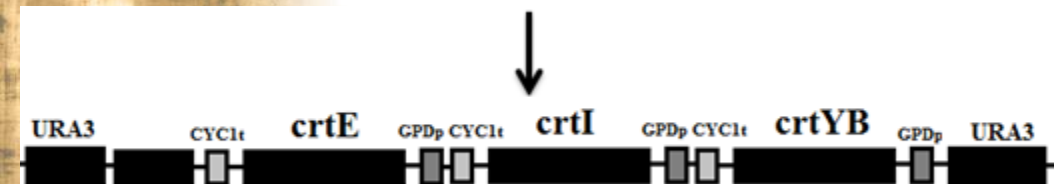
Stu I

5'... AGG CCT ... 3'
3'... TCC GGA ... 5'

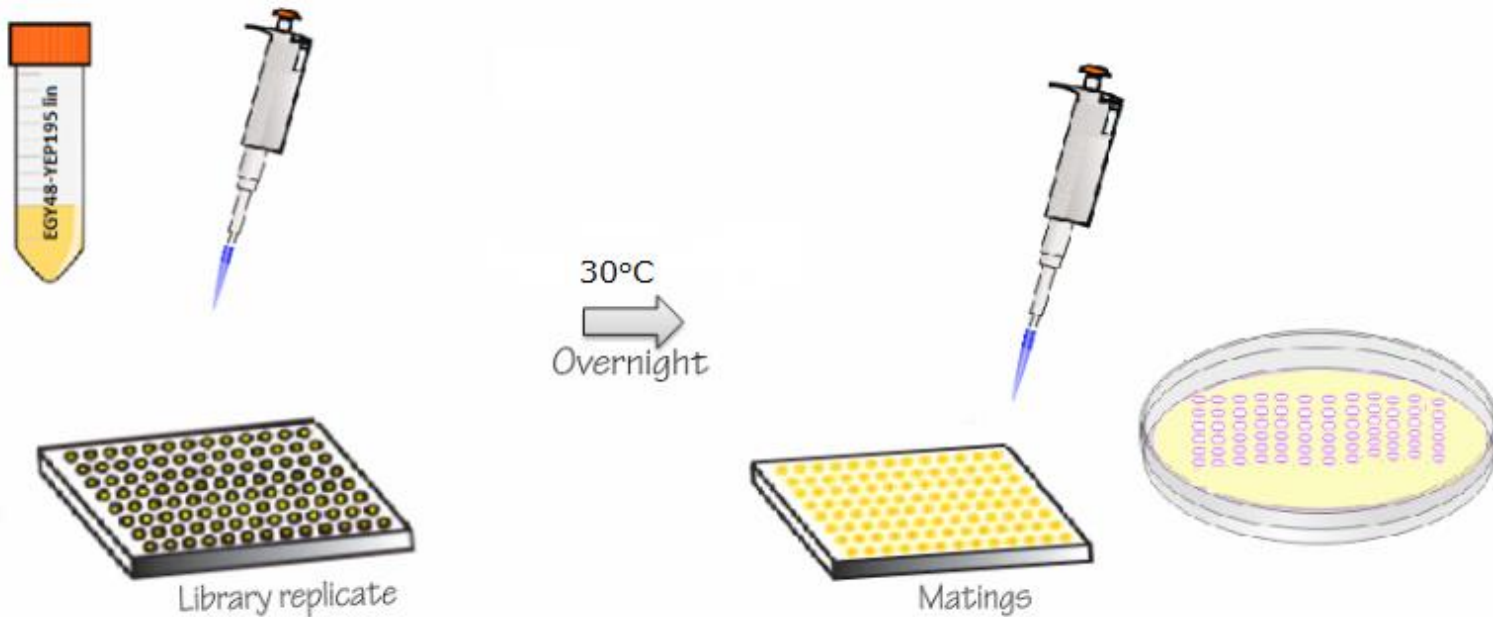


- Integration into a wild type Mat alpha yeast strain

Integration should eliminate plasmid instability issues during the screen



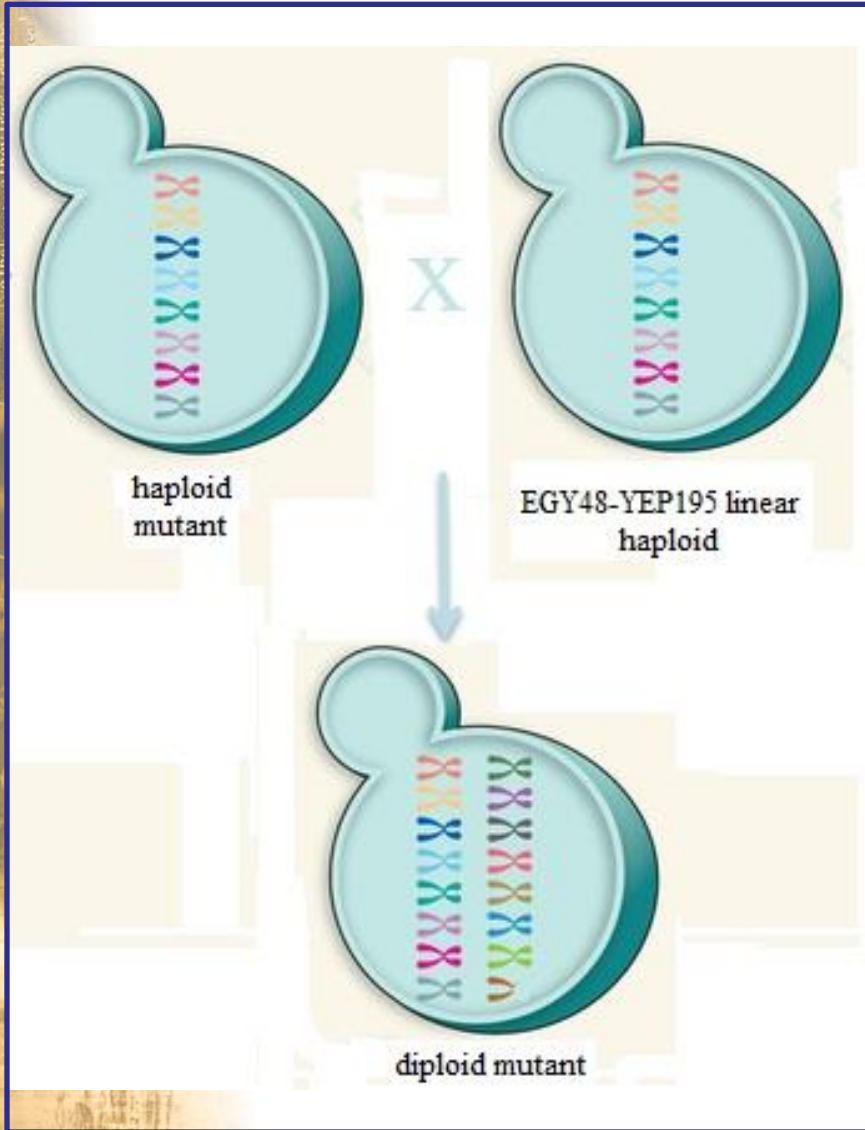
Mating of the Mat alpha, YEplac195-crtYB/crtI/crtE strain to the Research Genetics yeast deletion strain library



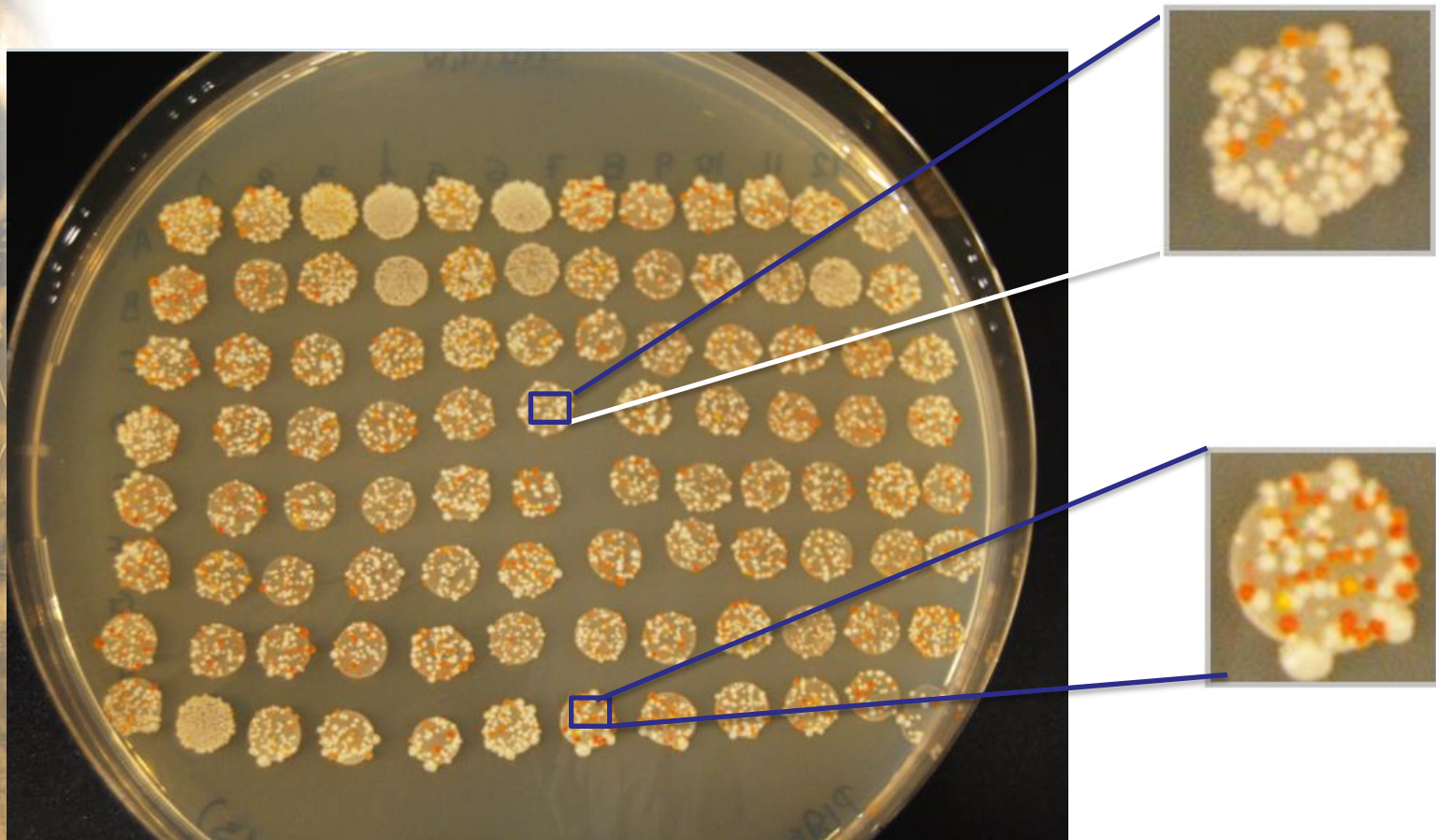
- Library (Research Genetics):
4250 deletion mutant strains

- Plating in
selection media
for diploid strains

Cross of library strains to Mat alpha carotenoid producing strain

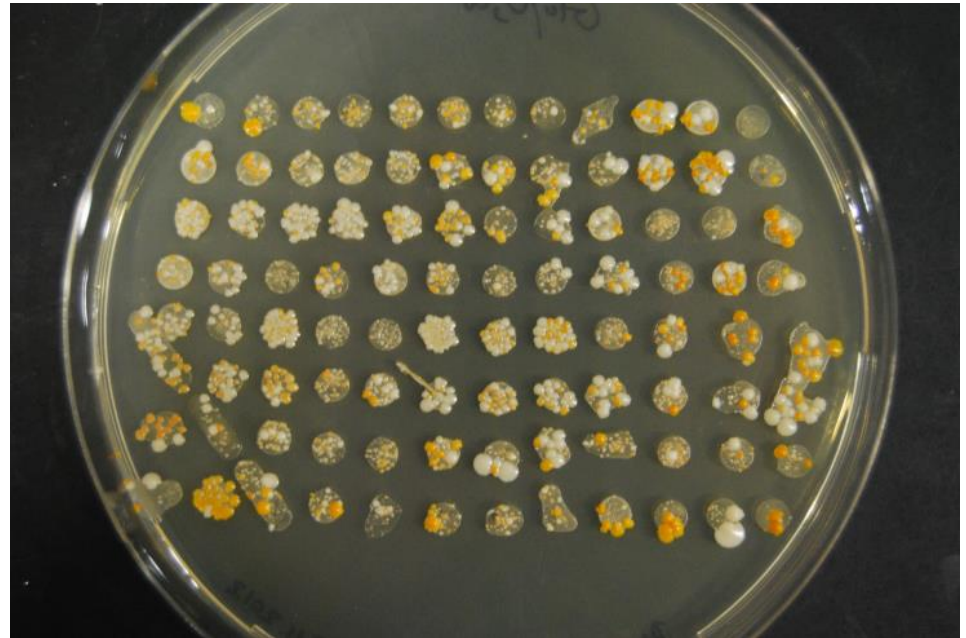


Diploid heterozygous library strains



- 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



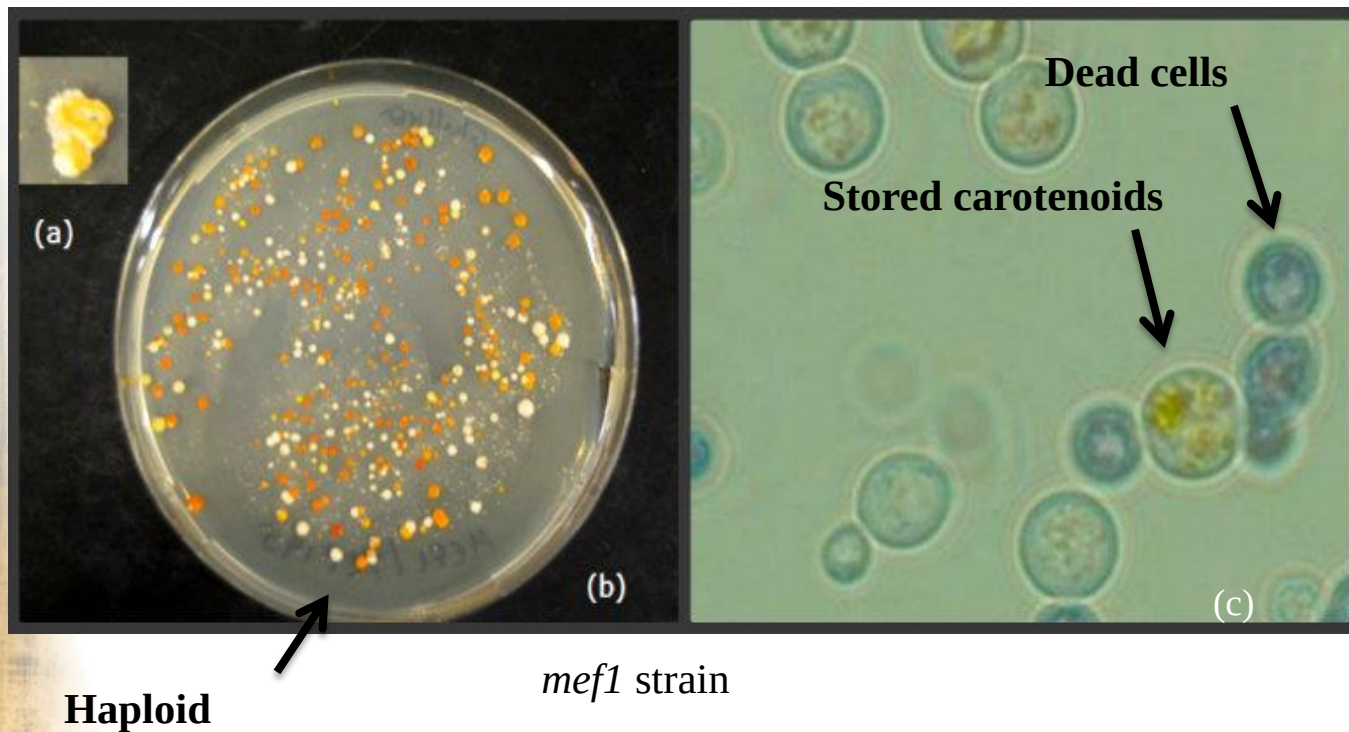


• To the selected set of deletions identified, the corresponding haploid library deletion strains, the following plasmids were transformed

- » 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



Quantification of carotenoids

yor292w

ipt1

rvs167

cdc40

ist1

rps19b



ict1

mei4

yor029w

mef1

mus81

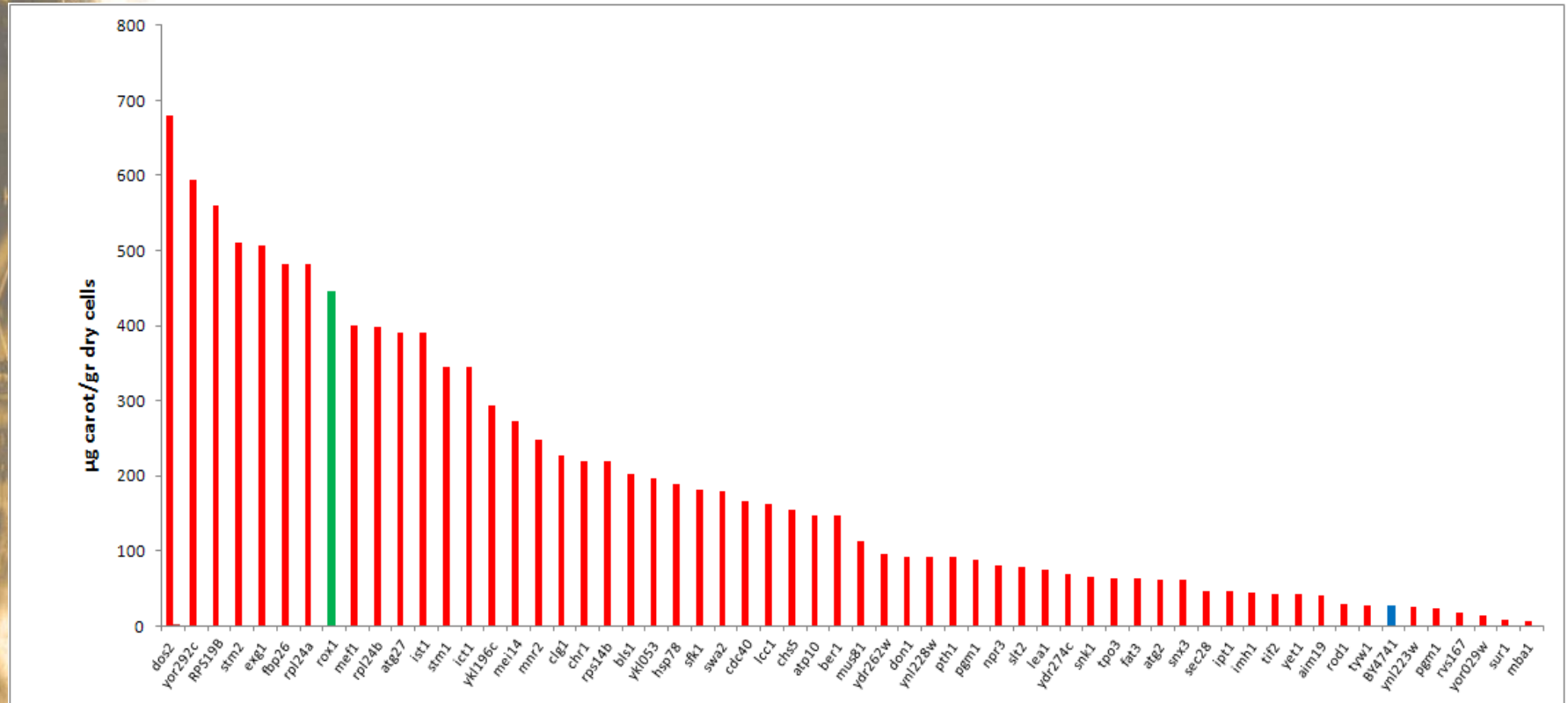


Selected colonies were used for inoculation of 50 ml cultures

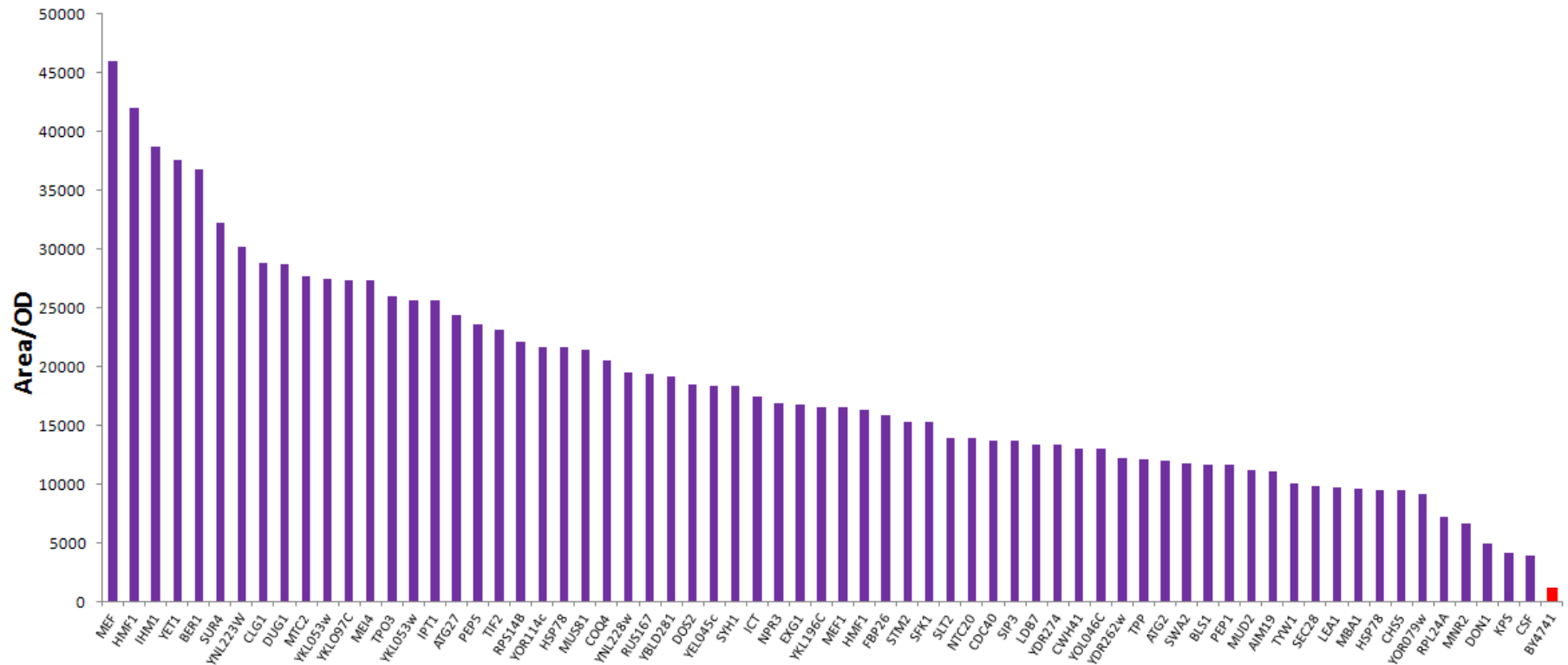


Cultures grown after 5 days incubation period

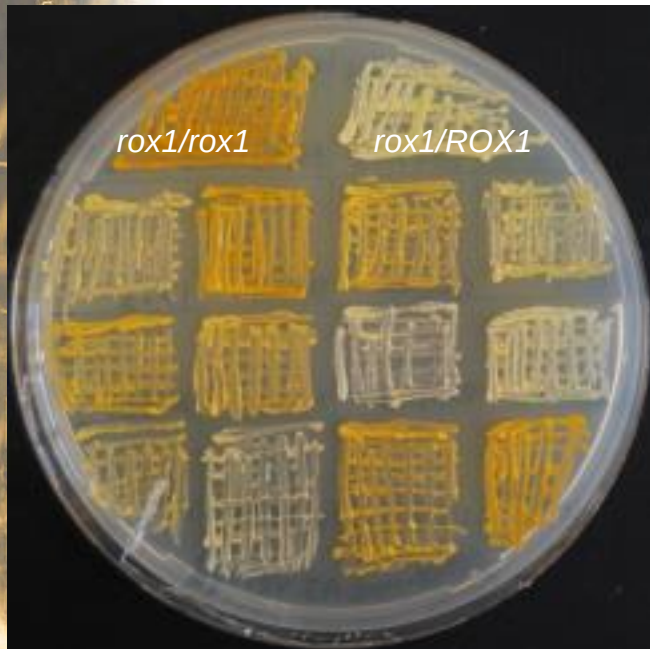
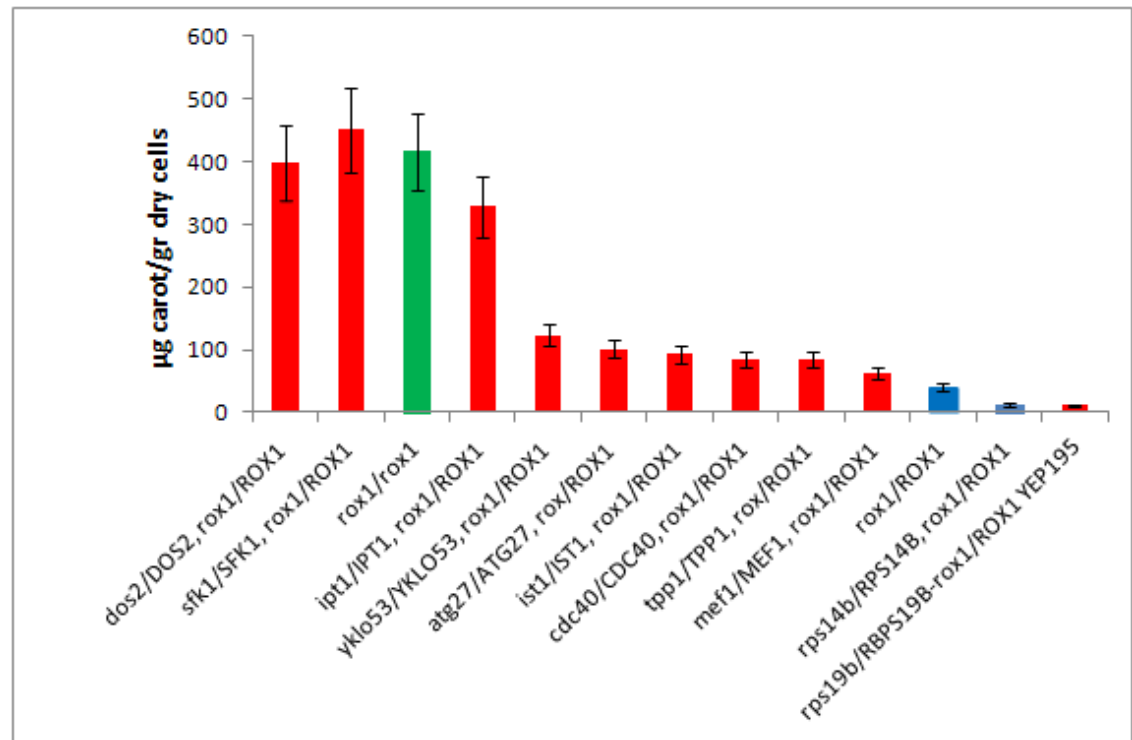
Carotenoid production of selected haploid mutants



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



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CERTH

Fotini Triikka

Anastasia Athanasakoglou

Alexandros Nikolaidis

Notis Argiriou

Antonios Makris



Dragana Bozic

Angelos Kanellis



CIHEAM

Codruta Ignea

Sotirios Kampranis

