Development of a visual screen for heterologously expressed plant diterpene synthases expressed in *Saccharomyces cerevisiae*

A dissertation submitted for the partial

fulfillment of BS-MS dual degree in Science

By

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Dedicated to my family

Certificate of Examination

This is to certify that dissertation titled **"Development of a visual screen for heterologously expressed plant diterpene synthases expressed in *Saccharomyces cerevisiae*"** submitted by Ms. Akanksha Jain (MS14021) for the partial fulfillment of BS-MS dual degree programme of the Institute, has been examined by the thesis committee duly appointed by the Institute. The committee finds the work done by the candidate satisfactory and recommends that the report is accepted.

Dr. Ram Yadav Dr. Kavita Babu Prof. Anand Kumar Bachhawat

(Supervisor)

Dated: April 24, 2019

Declaration

I hereby declare that the work presented in this dissertation entitled "**Development of a visual screen for plant diterpene synthases expressed in *Saccharomyces cerevisiae***" has been carried out by me under the guidance of Prof. Anand. K. Bachhawat at the Indian Institute of Science Education and Research Mohali. This work has not been submitted in part or in full for a degree, a diploma, or a fellowship to any other university or institute. In keeping with the general practice of reporting the scientific observations, due acknowledgment has been made wherever the work described is based on the finding of other investigators. This thesis is a bonafide record of original work done by me and all sources listed within have been detailed in the bibliography.

Akanksha Jain

Dated: April 24, 2019

In my capacity as the supervisor of the candidate's project work, I certify that the above statements by the candidate are true to the best of my knowledge.

Prof. Anand Kumar Bachhawat

(Supervisor)

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ABBREVIATIONS

Weights and measures

%	Percent
µmol,nmoles,mmoles,	micromole, nanomoles,millimoles,
°C	Degreecentigrade
bp, kb	Base pair,kilobase
kDa	Kilodalton
O.D.	Optical density
Psi	Pounds per squareinch
rpm	Revolutions perminute
RT	Roomtemperature
sec,min, h	Second, minute,hour
µg,mg,g	microgram, milligram,gram
µl, ml, L	Microliter, milliliter,liter
µM,mM, M	micromolar, millimolar, molar

Symbols

≈	Approximately
=	Equalto

Techniques

PCR	Polymerase ChainReaction

Chemicals

Amp	Ampicillin
ATP	AdenosineTriphosphate
dNTPs	2’-deoxyadenosine5’-triphosphate

EDTA	Ethylenediamine-tetra-aceticacid
HCl	HydrogenChloride
PEG	Poly EthyleneGlycol
PCI	Phenol- chloroform-isoamyl alcohol

Miscellaneous

DNA	Deoxyribonucleicacid
EUROSCARF	European *S. cerevisiae* Archive for Functional Analysis
LB	LuriaBertani
NCBI	*National Center for Biotechnology Information*
TE	Tris chloride andEDTA
WT	Wild-type

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Abstract

Terpenoids or Isoprenoids have uses in pharmaceuticals, agrochemicals, fragrances, synthetic rubber, and fuels. Terpenoids can be produced by metabolic engineering by expressing heterologous terpene synthases (TPSs) in bacteria and yeasts. Since both substrates and products of TPSs are colorless, diverse in structure, and mostly volatile, there is a need for screening system to screen for mutants with higher catalytic activity. The broad goal of the project aims to develop a visual carotenoid-based genetic screen in yeast to identify heterologously expressed superior catalytic variants of TPSs (specifically diterpene synthases) depending on the variation in the color intensity of the colonies. In the present study, towards this goal, lycopene biosynthesis enzymes were attempted to be integrated into yeast genome using the CRISPR/Cas9 system in a markerless integration strategy. Also, since the visual carotenoid-based screen is functional only in a small window, a delicate balance of carotenoid production concomitant with the diterpene production is needed. This aim has been targeted by making cassettes for all the genes under different strength of promoters.

Chapter 1

Introduction and Reviewof Literature

1.1 A brief introduction to terpenoids

Isoprenoids or terpenoids are secondary metabolites that are classically produced by all kingdoms of life including bacteria, fungi, and plants. They form one of the largest class of natural products with huge structural diversity and molecular functions that contribute more than 55,000 compounds to the rich chemical diversity of natural product structures available[1][2]. Based on the number of isoprene (C_5H_8) units present, they are classified into the following classes: monoterpenes ($C_{10}H_{16}$), sesquiterpenes ($C_{15}H_{24}$), diterpenes ($C_{20}H_{32}$), triterpenes($C_{30}H_{48}$), tetraterpenes ($C_{40}H_{64}$), and polyterpenes[$(C_5H_8)n$]. They include many commercially valuable compounds and are used in pharmaceuticals, agrochemicals, fragrances, synthetic rubber, and jet fuels[2].

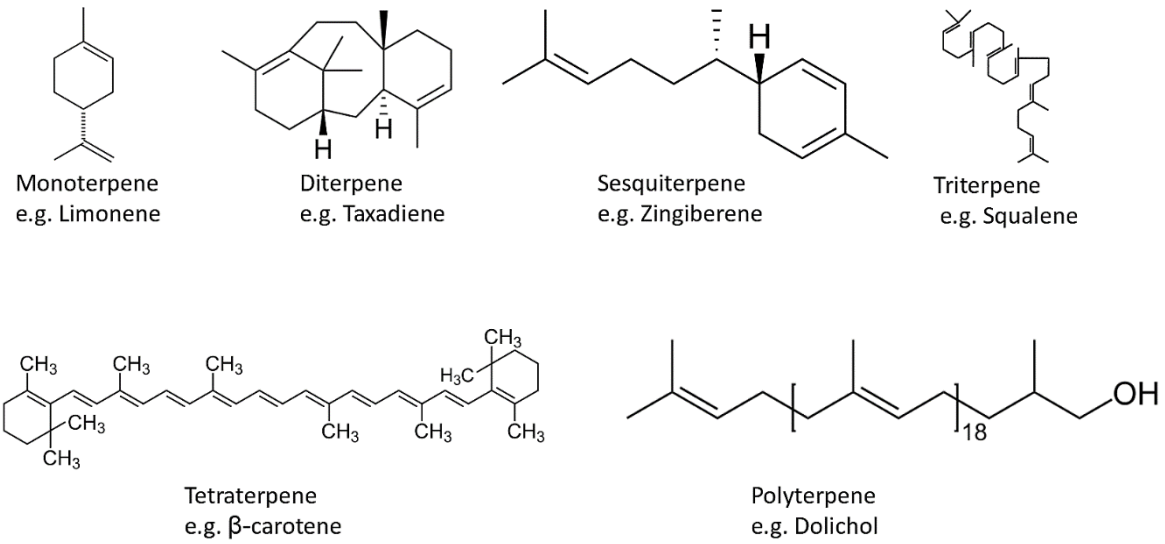


Figure1. Structures of different classes of terpenoids

1.2 Biosynthesis of Isoprenoids or Terpenoids

Terpenoids are biosynthesized from two C5 precursors- isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP)[1]. Two major pathways can produce the precursors for all the terpenoids- the mevalonate pathway (MVA) and the methyl-D-erythritol 4-phosphate (MEP) pathway.

The MVA pathway is the only pathway by which terpenoid precursors are produced in fungi

and animals including humans. The pathway commences with acetoacetyl-CoA (AA-CoA) that has been produced in the cytosol (not mitochondrial acetyl-CoA) and proceeds through five enzymatic steps via the intermediate mevalonate (MEV) to IPP. IPP is isomerized to DMAPP by IPP isomerase, and the two molecules are combined to form larger isoprenoids, such as farnesyl diphosphate (FPP)[3].

The MEP(Methyl erythritol phosphate) pathway is mainly employed in most eubacteria and all photosynthetic eukaryotes, cyanobacteria, and plants. The biosynthesis of IPP and DMAPP takes place in plastid organelles. This pathway is absent in animals, fungi and archaebacteria. It comprises of seven enzymatic steps. MEP transforms to the cytidine 5-diphosphate derivative and sequential phosphorylation at C, and cyclization leads to 2-C-methylerythritol-2,4-cyclodiphosphate. Loss of CMP forms MEcycPP which is further converted to 1-hydroxy-2methyl-2-(E)-Butenyl 4-diphosphate(HMBPP). The last step is a branching step where HMBPP is converted to mixture of IPP and DMAPP[4].

Plants have two pathways, the MEP pathway which occurs in plastids and the MVA pathway which occurs in the cytosol.

1.3 Metabolic engineering of Terpenoids in yeast

Limited production of terpenoids from natural sources, challenging extraction procedures and high cost involved in chemical synthesis makes this topic unique to study [5].Many metabolic engineering efforts have been undertaken in the past to increase the levels of terpenoids in the heterologous hosts such as *Escherichia coli* and *Saccharomyces cerevisiae.* Therefore, if the biosynthetic pathways are known, the metabolic engineering of microorganisms for the production of terpenoids provides a relatively fast and inexpensive route, and that can result in purer product with a higher yield. The yeast, *S. cerevisiae* has appeared as one of the preferred host organisms for the production of isoprenoids by the industry due to the relatively high native isoprenoid/mevalonate pathway flux, ease of genetic manipulations, GRAS (generally regarded as safe), knowledge of its genetic and physiological characteristics and availability of engineering tools[6]. Furthermore, many higher eukaryotic genes express well in *S. cerevisiae.*

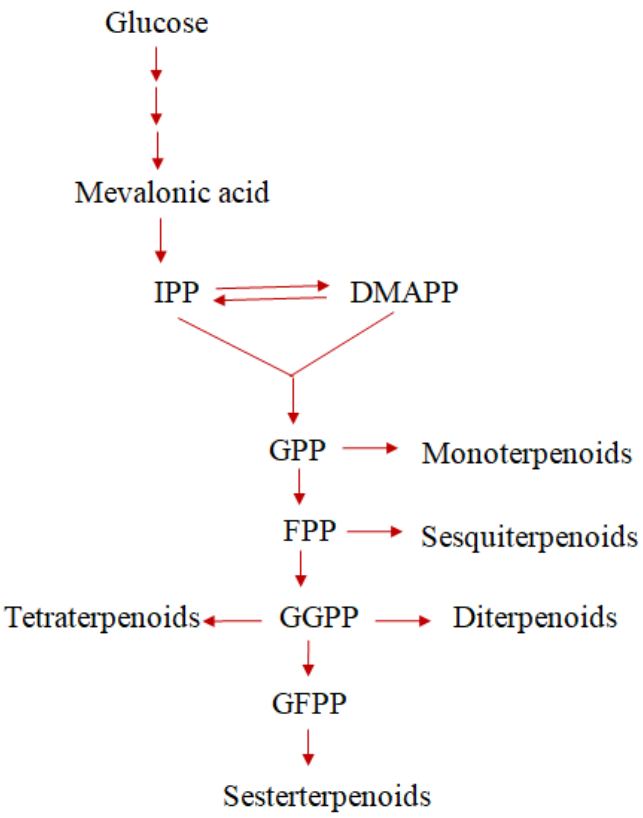


Figure2. Schematic representation of the production of different terpenoids from different precursors of the mevalonate pathway in yeast

1.4 Metabolic engineering of *S. cerevisiae* for Carotenoids production

Carotenoids are colored tetraterpenoids that are classically produced in nature by plants and many microorganisms including bacteria, fungi, and algae. Animals can metabolize carotenoids, but they cannot synthesize. The carotenoids consist of eight isoprenoid rings resulting in a C_{40} backbone. Carotenoids primarily produce yellow, red and orange colors and are the most widely distributed pigments. They are used as food,nutraceuticals, cosmetic color additives and used widely in many industrial applications. Carotenoids also have provitamin A activity, as this particular vitamin is a product of carotenoid metabolism[7][8]. Anti-aging and anti-oxidant activity have also been reported for carotenoids[9].

Carotenoid biosynthesis can be engineered in *S. cerevisiae*. In metabolic engineered *S. cerevisiae* cells, three carotenogenic enzymes geranyl geranyl pyrophosphate synthase (GGPPS), phytoene synthase (PS) and phytoene dehydrogenase (PD) from the carotenogenic organisms are heterologously expressed to produce β-carotene. GGPPS catalyzes the

conversion of farnesyl diphosphate (FPP) to form geranyl geranyl pyrophosphate (GGPP) which is further converted into phytoene by the bifunctional enzyme phytoene synthase. Subsequently, phytoene is desaturated by phytoene desaturase (dehydrogenase) to form Lycopene. Lycopene also undergoes several metabolic reactions by the action of the bifunctional enzyme phytoene synthase to produce γ-carotene and β- carotene[3].

Schematic representation of the heterologous expression of carotenogenic enzymes of *Rhodosporidium toruloides* for the production of carotenoids from the mevalonate pathway of *Saccharomyces cerevisiae* is shown in figure 3.

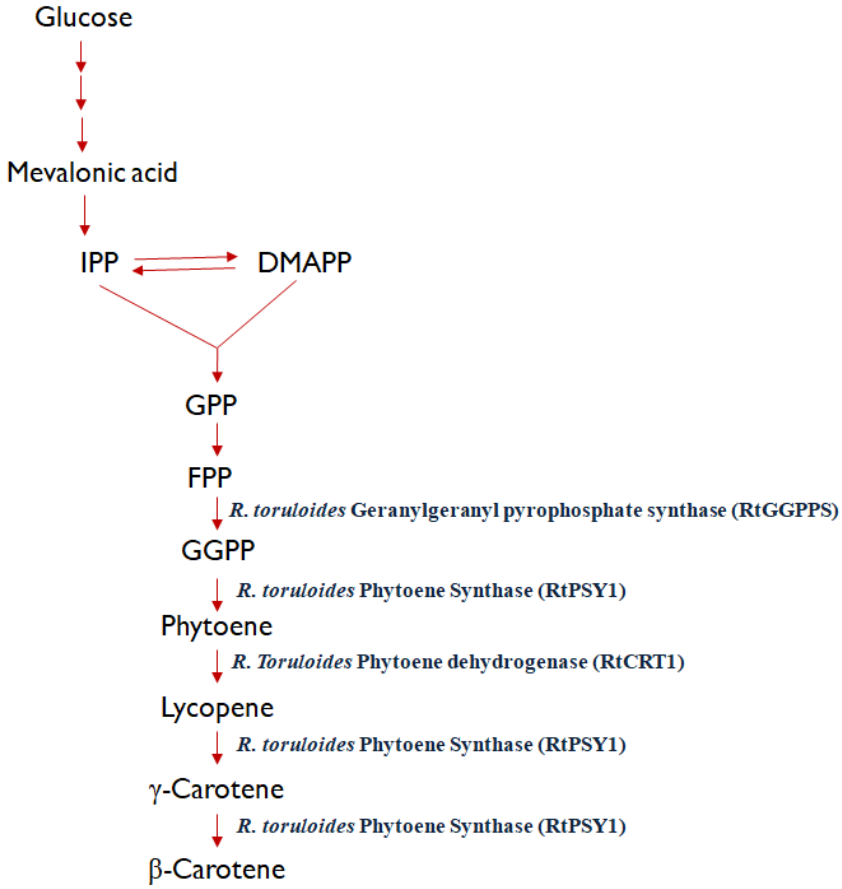


Figure3. Schematic representation of the heterologous expression of carotenogenic enzymes of *Rhodosporidium toruloides* for the production of carotenoids from the mevalonate pathway of *Saccharomyces cerevisiae*

1.5 Carotenoid-based substrate consumption assay for terpenoids

There is a lack of high throughput screening system for screening the activity of terpene synthases (TPSs) since both the substrates and products of TPSs are colorless, diverse in structure, and mostly volatile[2]. Since Carotenoids and Terpenoids use the same isoprenyl diphosphate (FPP and GGPP) as substrates[10], expression of active TPSs results in decreased availability of building blocks for carotenoid biosynthesis reducing pigmentation of the host cells. So, the activity of any TPS can then be rapidly detected by assessing the variation in the color intensity of the colonies[2]. The carotenoid-based screening system is based on substrate consumption rather than the detection of a specific product. Therefore, this is applicable to any TPSs irrespective of the product type. This assay provides many advantages such as visualization of the landscape of the mutant libraries, rapid isolation of the active terpenoid genes from inactive ones, search the variants for improved activities, and Substrate-size-specific screening for monoterpene synthase (monoTPS), sesquiterpene synthase (sesquiTPS) and diterpene synthase (diTPS) activities can be done. M. Furubayashi *et al.*developed this screening method for the cellular activities of terpene synthases when heterologously expressed in *E.coli*. This strategy had been utilized for improving the performance of taxadiene synthase (TXS), geraniol synthase (GES) and tobacco 5-epi-aristolochene synthase (TEAS) byscoring their substrate consumption based on the color loss of the cell harboring carotenoid pathways. The advantage of the carotenoid-based approaches is that it allows the screening of whole cells. One disadvantage of using these screening methods is the high rate of false positives during the selection of loss-of-function mutations for improving the isoprenoid synthases. Decreased pigmentation in loss of function mutant strains would not be necessary due to a positive effect on the isoprenoid biosynthesis activity; it could be due to factors that reduced cell viability.

1.6 Sclareol

Our lab has been interested in improving an engineered pathway for sclareol biosynthesis in yeast. Sclareol is a naturally occurring labdane type bicyclic terpene alcohol. It is found in some plants, e.g., Clary Sage (*Salvia sclarea*), Nicotiana species, etc.). It is an amber colored solid with a sweet, woody odor. It is extensively used as starting material for the synthesis of

fragrance molecules with ambergris notes (e.g., Ambrox). Presently the primary sources of sclareol are flowers and leaves of *Salvia sclarea* (clary sage), and lesser yields are obtained from several other plants, e.g., *Nicotiana tabacum.* Sclareol biosynthetic pathway using enzymes from Salvia and Nicotiana species and their various genetic modifications have been engineered in *Escherichia coli* and *Saccharomyces cerevisiae*[14], and has also been patented recently by two companies: Firmenich (US Patent 8,586,328 B2; US Patent,8,617,860 B2 and US Patent 9,267,155 B2) and Evolva (US Patent 2016 9,353,385 B2) for sclareol production.So, there is a need to improve the catalytic efficiency of these enzymes.

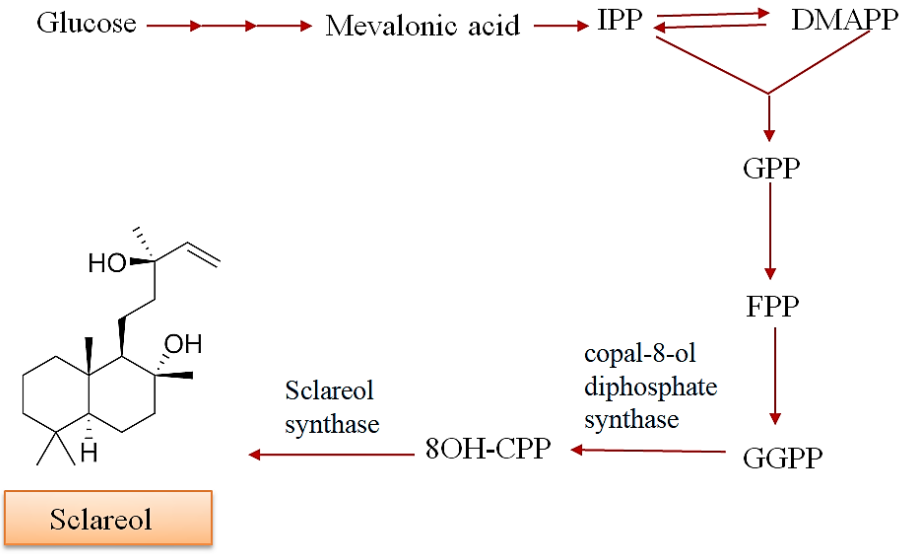


Figure4. Schematic representation of the biosynthetic pathway of sclareol production whenplant genes are heterologously expressed in yeast

1.7 Assembling of genetic tools required for the optimization of substrate consumption assay in yeast

The visual genetic screen for screening the higher catalytic mutants for sclareol production needs the genes under different strength of promoters for two pathways:

1. Carotenoid pathway producing colored carotenoids like β-carotene and lycopene-
   a) GGPPs - geranyl geranyl pyrophosphate synthase
   b) PS - Phytoene Synthase
   c) PD - Phytoene desaturates

2. Diterpene (sclareol) biosynthetic pathway:
    a) SsSS - sclareol synthase from *Salvia sclarea*
    b) CcCLs - copal-8-ol diphosphate synthase from *Cistus creticus*

Previously, the genes of the carotenoid pathway RtGGPPS, RtPSY1, and RtCRT1 were expressed in *S. cerevisiae* strain (Fig.5A). Expression of the sclareol biosynthesis genes into this strain requires multiple plasmids and resulted in various color colonies consisting of white, orange and pale (Fig. 5B and 5C) which is incomparable for the screen.

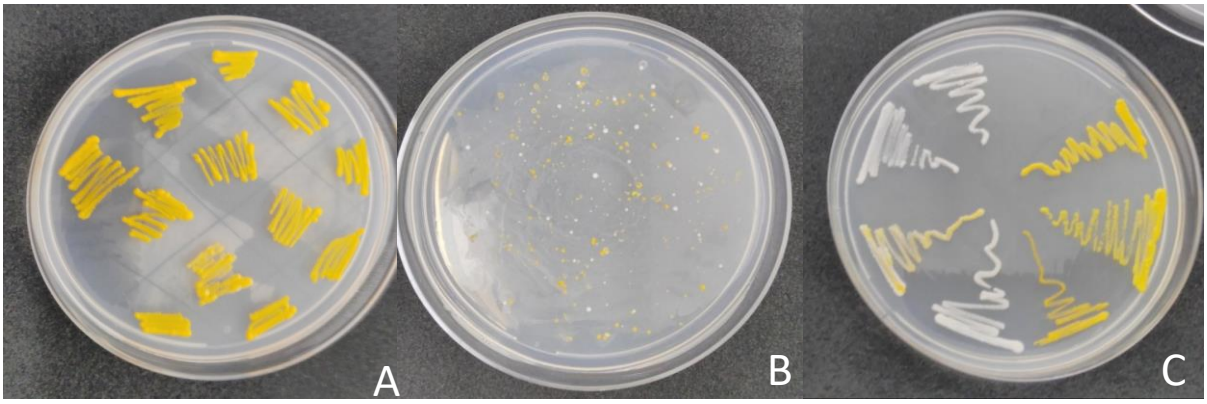


Figure 5. (A) *S. cerevisiae* ABC276 strain transformed with (RtCRT1+RtPSY1) recombinant plasmid and RtGGPP synthase, orange color colonies observed (B) CcCLS/pRS313TEF construct and SsSS/p416TEF transformed in the above *S. cerevisiae* strain (C) Non-homologous landscape of transformants

The above scenario points to the need for further optimization on the following aspects:

a) Stable genetic integration of the three carotenogenic genes is required since five genes need to be expressed into yeast, using plasmids leads to lower efficiency.
b) Stable integration of carotenogenic genes should be markerless and at non-essential genes keeping the auxotrophic markers available for expressing the recombinant constructs having diterpene synthase genes.
c) Also, there is a need to maintain a delicate balance between the carotenoids production with the diterpene production as the substrate consumption assay is likely to be functional only in a small window. So, there is a need to make constructs for all of the genes under different strength of promoters.

1.8 Lycopene producing *S. cerevisiae* strain

Lycopene producing *S. cerevisiae* strain has been previously created in the lab which leads to the production of red colored lycopene only and gives better pigmentation and hence, has better ability to lead to a better visual screen. For heterologously producing lycopene in *S. cerevisiae*, in addition to RtGGPPS and RtCRT1, the phytoene synthase used is *Arabidopsis thaliana* phytoene synthase (AtPS). AtPS differs from RtPSY1 in being monofunctional and lacking the cyclase domain thereby instead of producing the cyclic carotenoids like β-carotene, it can only produce a linear chain carotene like lycopene and limits the pathway to produce a minimum number of colored metabolites to one, i.e., lycopene. Schematic representation of the biosynthetic pathway of lycopene production whencarotenogenic genes are heterologously expressed in *S. cerevisiae* has been shown in figure 6.

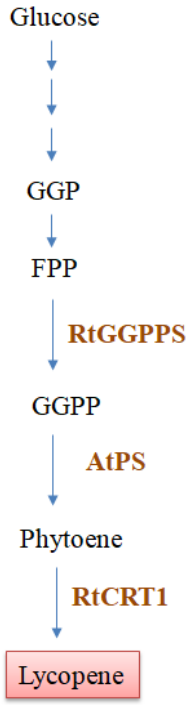


Figure 6. Schematic representation of the biosynthetic pathway of lycopene production whencarotenogenic genes are heterologously expressed in yeast

Therefore, a lycopene producing strain is a better choice for developing a visual screen for

screening plant diterpene synthases based on the substrate consumption assay.

Schematic representation of the heterologous expression of carotenogenic enzymes to produce lycopene followed by the expression of plant diterpene synthases for the sclareol production which leads to the development of substrate consumption assay in *S. cerevisiae* is shown in figure 7.

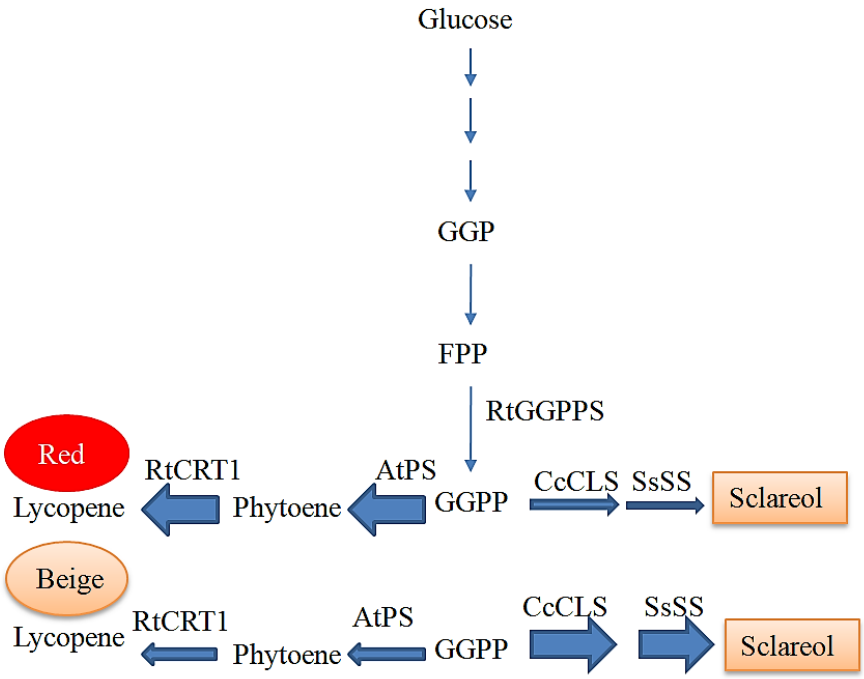


Figure 7. Schematic representation of the substrate consumption assay in lycopene producing *S. cerevisiae* strain when plant diterpene synthases are heterologously expressed

1.9 Strategies for markerless genomic integration of heterologous genes in *S. cerevisiae*

Expression and optimization of heterologous metabolic pathways in *S. cerevisiae* require the introduction of successive multiple genetic modifications including the integration of the genes at single/multiple loci to generate the metabolic product [17]. In addition to being a very time-consuming and labor-intensive process, the maximum number of sequential modifications are

limited by the availability of the selection markers. Many strategies for the recycling of the genetic markers have been developed, such as homologous recombination-mediated counter selection and generation of I-SceI-induced double-stranded break (DSB)[18]. These methods largely eliminate the limitations by marker-gene availability, but they are still remain a time-consuming process. Alternative methods such as zinc finger nucleases (ZFNs)[19] and transcription activator-like effector nucleases (TALENs)[20] utilize DSB for site-directed genomic editing, but for these methods also, a new ZFN and TALEN is required for each genetic modification. More recently, an alternate genome editing approach based on the CRISPR-Cas9 system (clusters of regularly interspaced palindromic repeats-CRISPR associated nuclease 9, has emerged[21]. The present work aims to use this strategy for markerless integration of heterologous genes in *S. cerevisiae*.

1.10 CRISPR-Cas9 system for genomic editing

In bacteria and archaea, CRISPR–Cas9 system is originally a part of adaptive immunity against invading foreign nucleic acids[22]. *Streptococcus pyogenes* Cas9 is an endonuclease which complexes with two small RNA molecules to form ribonucleoprotein complex and performs sequence-specific cleavage of the invading DNA[23]. It has been shown that Cas9 can function as an RNA-guided endonuclease in heterologous organisms[24]. CRISPR systems offer an advantage to zinc finger and transcription activator-like effector DNA binding proteins, as the site specificity in nucleotide binding CRISPR-Cas proteins is governed by the RNA molecule instead of the DNA-binding protein, which is more challenging to design and synthesize[24].

In 2013, DiCarlo *et al.* (2013b) employed the CRISPR/Cas9 system for the introduction of DSBs in *S. cerevisiae*. In a strain expressing a plasmid-borne human codon-optimized cas9 gene from *S. pyogenes*, a second plasmid was transformed, containing the SNR52 promoter followed by a sequence encoding for guide-RNA (gRNA) with a 20 bp targeting sequence for CAN1(A plasma membrane Arginine permease and its mutation confer canavanine resistance). Cas9 did bind and make disruption at the 20 bp target sequence (CAN1 specific) on being guided by gRNA to that site on condition that a proper protospacer adjacent motif (PAM) is

proximally adjacent to it (in case of *S. cerevisiae*, NGG is the PAM sequence)[22]. Subsequently, this otherwise lethal break was repaired by the yeast Homologous Recombination (HR) machinery, using a co-transformed repair fragment that bridged the flanking regions of the break. The repair fragment used was designed to introduce a premature stop codon, whose integration at the DSB region of CAN1 locus resulted in colonies that were resistant to canavanine[21][25]. This strategy and its modified versions have been recently used for introducing genetic modifications in yeast by numerous research group[25][26][27].

Rarely, non-homologous end-joining (NHEJ) can generate mutations which block Cas9 cleavage despite failing to incorporate the expected genomic alterations. In such cases, cells merely succumb to the stress of repeated Cas9-induced genomic cleavages. In an appropriately conducted experiment, the majority of the surviving transformants are those that have lost their CRISPR target site by incorporating the desired genomic alteration via HR. Cas9 thus acts as a counter-selection acting directly on the genomic sequence, rather than its phenotypic manifestations[28].

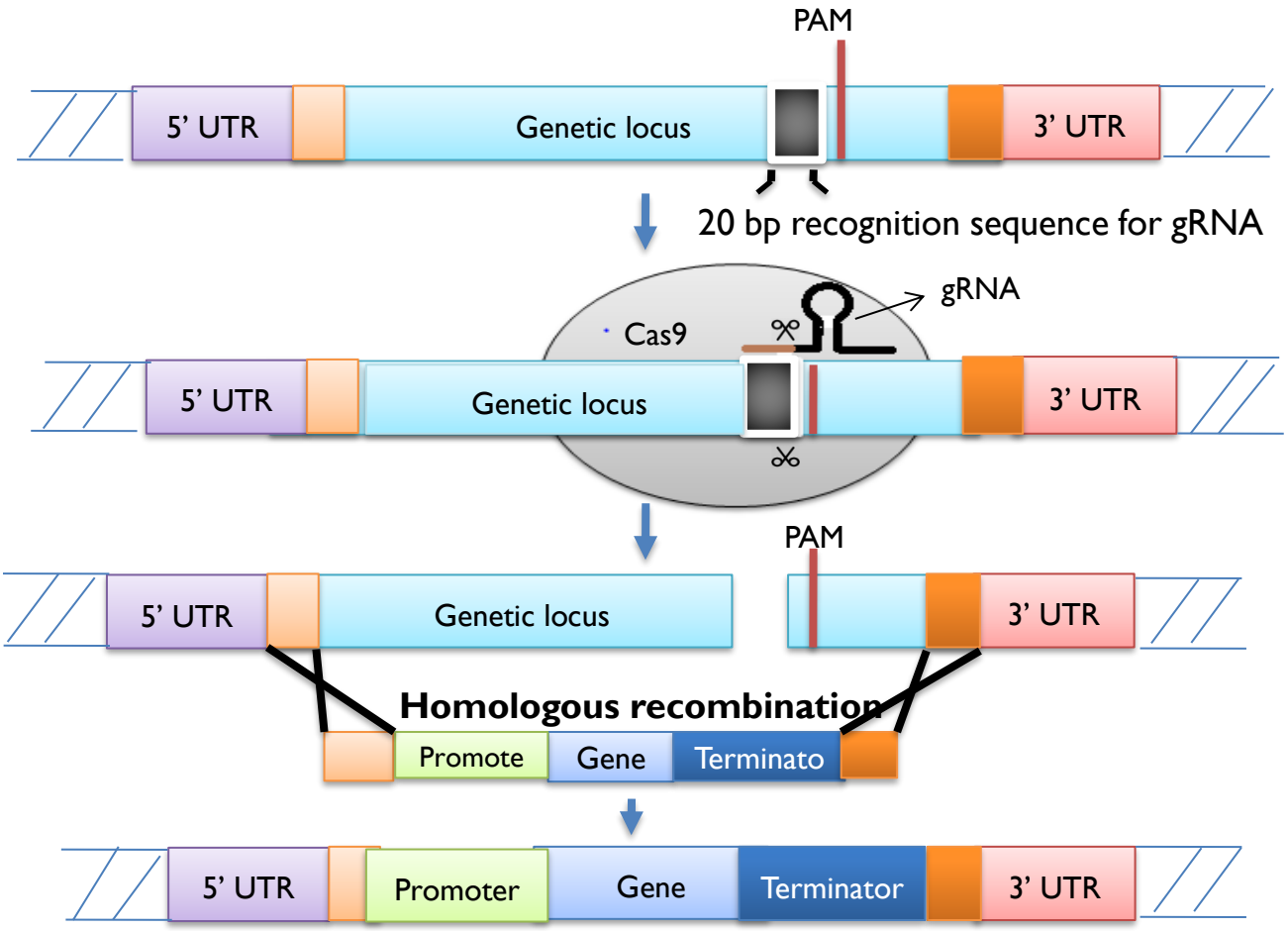


Figure 8. Schematic representation of genomic integration of the "gene of interest" at the targeted locus by CRISPR-Cas9 system in yeast

1.11 Objective of the study

The principal goal of this study was to develop a visual carotenoid-based genetic screen in yeast *S. cerevisiae* to identify heterologously expressed superior catalytic variants of diTPSs. More specifically, we have listed out the following objectives –

1. To integrate genes for lycopene biosynthesis in *S. cerevisiae* genome using CRISPR-

Cas9

2. Express the diterpene synthase enzymes for sclareol biosynthesis (CcCLS and SsSS) downstream promoters of various strength
3. To evaluate the strain producing lycopene in a substrate consumption assay to obtain improved variants of sclareol biosynthesis enzymes – 1) CcCLS 2) SsSS

Chapter 2

Materials and Methods

SECTION A: MATERIALS

2.1 CHEMICALS ANDREAGENTS

All the chemicals used in this study were obtained from commercial sources and were of analytical grade. Media components, fine chemicals, and reagents were purchased from Sigma Aldrich, (St. Louis, USA), HiMedia, (Mumbai, India), Merck. India Ltd (Mumbai, India), USB Corporation (Ohio, USA) or Difco, USA. Oligonucleotides (primers) were designed using Snapgene software and were purchased from Integrated DNA Technologies (IDT) or Sigma Aldrich (Bangalore, India). Enzymes (Restriction enzymes, T4 DNA ligase, Calf Intestinal Phosphatase (CIP), Antarctic phosphatase, *Vent* DNA polymerase, *Taq* DNA polymerase, Phusion® DNA polymerase, and other modifying enzymes), their buffers and dNTPs were purchased from New England Biolabs Inc, (Beverly, MA, USA). Gel-extraction kits and plasmid miniprep columns were obtained from Bioneerand Promega.

2.2 STRAINS ANDPLASMIDS

Escherichia coli DH5α was used as the cloning host. The genotype for the *E. coli* strain and the yeast strains used in this study are given in Table 1. The list of various plasmids used in this study is given in Table 2.

Table 1: List of bacterial and yeast strains used in the study

Strain	Genotype	Source
Escherichia coli strain		
ABE 460 (DH5α)	*F- gyrA96*(Nal) *recA1 relA1 endA1 thi-1 hsdR17* (rk -m k+) *glnV44 deoRΔ(lacZYA-argF) U169* [ϕ80dΔ*(lacZ) M15*]	Lab strain
***Saccharomyces cerevisiae* strains**		
ABC 276	*MATα,ura3-52, leu2Δ1, his3Δ200,trp 1,lys 2-801*	Lab strain
ABC 5001 (IMX672) [25]	*MATa ura3-52 trp1-289 leu2-3,112 his3 can1::cas9-natNT2*	Euroscarf

Table 2: List of Plasmids used in the study

Plasmid name	Clone no.	Description
pRS416TEF	ABE 443	The CEN-vector bearing URA3 marker and TEF promoter-MCS-terminator for yeast expression and Amp^r marker for selection in *E. coli*[29]
pRS426GPD	ABE 446	The CEN-vector bearing URA3 marker and GPD promoter-MCS-terminator for yeast expression and Amp^r marker for selection in *E. coli*.
pRS416GAL1	ABE 449	The CEN-vector bearing URA3 marker and GAL1 promoter-MCS-terminator for yeast expression and Amp^r marker for selection in *E. coli*.
pRS414-GAL1-GCN4	ABE 2152	The CEN-vector bearing *TRP1* marker and GAL1 promoter-MCS-terminator for yeast expression and Amp^r marker for selection in *E. coli*. The plasmid was obtained from Daniel Kornitzer MCB, 22,2002. / EcoR1-Xho1.

pRS315TEF	ABE 3488	The CEN-vector bearing LEU2 marker and TEF promoter-MCS- terminator for yeast expression and Amp^r marker for selection in *E. coli*. Vector was constructed by excising TEF promoter and MCS from pRS416TEF using *SacI* and *ApaI* site and cloned at pRS315 vector at *SacI* and *ApaI* site.
pRS314TEF	ABE 3498	The CEN-vector bearing *TRP1* marker and TEF promoter-MCS- terminator for yeast expression and Amp^r marker for selection in *E.coli.* Vector was constructed by excising TEF promoter and MCS from pRS416TEF using *Sac*I and *Apa*I site and cloned at pRS314 vector at *Sac*I and *Apa*I site.
pRS314TEF-CRTI	ABE 3500	Codon-optimized RtCRTI gene (phytoene dehydrogenase) of *Rhodosporidium toruloides* was cloned at *BamHI* and *SalI* site of pRS314TEF.
pRS315TEF-GGPPS	ABE 3518	Codon-optimized RtGGPPS gene (GGPP synthase) of *R. toruloides* was cloned at *XbaI* and *BamHI* site of pRS315TEF.
pRS416TEF(RtPSY1+ RtCRT1)	ABE 3551	The CEN-vector bearing URA3 marker and GAL1 promoter-MCS- terminator for yeast expression containing (RtPSY1+RtCRT1) gene from *R. toruloides* and Amp^r marker for selection in *E. coli*.
pRS313TEF	ABE 3569	The CEN-vector bearing *HIS3* marker and TEF promoter-MCS-terminator for yeast expression and Amp^r marker for selection in *E. coli.* Vector was constructed by excising TEF promoter and MCS from pRS416TEF using *Sac*I and *Apa*I site and cloned at pRS313 vector at *Sac*I and *Apa*I site.
pRS313TEF-GGPPS	ABE 3572	Codon-optimized RtGGPPS gene (GGPP synthase) of *R. toruloides* was cloned at XbaI and BamHI site of pRS313TEF.
pRS315CYC-GGPPS	ABE3633	TEF promoter (407bp) in pRS315TEF-GGPP (ABE 3572) was excised using *SacI* and *XbaI* and replaced by CYC promoter (300 bp) at *SacI* and *XbaI* site.
pRS314CYC- CRTI	ABE 3649	TEF promoter (407bp) in pRS314TEF-CRTI (ABE 3500) was excised using *Sac*I and *Bam*HI and replaced by CYC promoter (300 bp) at *Sac*I and *Bam*HI site.
pRS416CYC-PSY1	ABE3650	TEF promoter (407bp) in pRS416TEF-PSY1 (ABE 3519) was excised using *Sac*I and *Bam*HI and replaced by CYC promoter (300 bp) at *Sac*I and *Bam*HI site.

pRS315CYC-GGPPS	ABE3633	TEF promoter (407bp) in pRS315TEF-GGPP (ABE 3572) was excised using *SacI* and *XbaI* and replaced by CYC promoter (300 bp) at *SacI* and *XbaI* site.
pRS314CYC- CRTI	ABE 3649	TEF promoter (407bp) in pRS314TEF-CRTI (ABE 3500) was excised using *Sac*I and *Bam*HI and replaced by CYC promoter (300 bp) at *Sac*I and *Bam*HI site.
pRS313TEF- CRTI	ABE 3904	CRT1 gene in pRS314TEF-CRTI (ABE 3500) was excised using *Sal*I and *Bam*HI and ligated in vector pRS313TEF at *Sac*I and *Bam*HI site.
pRS416TEF-PS(At)	ABE 4159	*At*PS (phytoene synthase) from *Arabidopsis thaliana* cloned at *Bam*HI and *Xho*I site of pRS416TEF.
pRS315TEF-tHMG1	ABE 4740	HMG1 gene of *S. cerevisiae* is cloned without N-terminal (1575bp) at *BamHI* and *XmaI* site of pRS315TEF.
pRS313TEF -CRTI	ABE 4869	Codon-optimized *Rt*CRTI gene (phytoene dehydrogenase) of *Rhodosporidium toruloides* was cloned at *Bam*HI and *Sal*I site of pRS313TEF.
PMEL16 [25]	ABE 5227	p426-SNR52p-gRNA.CAN1.Y-SUP4t HIS3 based Yeast/*E.coli* shuttle vector designed to clone and express Cas9 programming spacer and has Amp^r marker for selection in *E. coli.*
PMEL17 [25]	ABE 5228	p426-SNR52p-gRNA.CAN1.Y-SUP4t TRP1 based Yeast/*E.coli* shuttle vector designed to clone and express Cas9 programming spacer and has Amp^r marker for selection in *E. coli.*
pRS315GPD CRTI_A393T	ABE 5245	*Rt*CRTI (phytoene dehydrogenase) gene of *R. toruloides* with A393T mutation and cloned in pRS315GPD.
pRS313TEF-CcCLS	ABE 5623	Codon-optimized gene CcCLS (copal-8-ol diphosphate synthase) of *Cistus creticus* was cloned at *Eco*RI and *XbaI* site of pRS313TEF.
pRS416TEF-SsSS	ABE 5624	Codon-optimized SsSS gene (sclareol synthase) of *Salvia sclarea* was cloned at *XhoI* and *BamHI* site of pRS416TEF.
pUC57-SiCPS	ABE 5687	Custom synthesized and codon-optimized SiCPS gene in vector pUC57 at *Eco*RI and *XbaI* site.
pUC57-SiTPS	ABE 5789	Custom synthesized and codon-optimized SiTPS gene in vector pUC57 at *XhoI* and *BamHI* site.

pRS416 {TEF-PS(At)+TEF-PD}-CYC	ABE 5856	Vector was constructed by amplifiying TEF-PD with CYC terminator and then cloned at the *KpnI* site of from pRS416TEF-PS(At) (ABE 4159).

Table 3: List of Plasmids constructed in the study

Plasmid name	Clone no.	Description
pRS416GPD	ABE 5800	The CEN-vector bearing *URA3* marker and GPD promoter-MCS-terminator for yeast expression and Amp^r marker for selection in *E.coli*. Vector was constructed by excising GPD promoter using *Sac*I and *Xba*I site from pRS426GPD (ABE 446) and cloned at pRS416-TEF(ABE 443) vector at *Sac*I and *Xba*I site.
pRS313GPD-CcCLS	ABE 5801	TEF promoter (407bp) in pRS313TEF-CcCLS (ABE 5623) was excised using *Sac*I and *Bam*HI and replaced by GPD promoter at *Sac*I and *Xba*I site.
pRS426GPD-SsSS	ABE 5803	SsSS gene from pRS416TEF-SsSS (ABE 5624) was excised using *XhoI* and *BamHI* site and then cloned in vector pRS426GPD (ABE 446) at *XhoI* and *BamHI* site.
pRS315GPD-tHMG1	ABE 5808	tHMG1 gene from pRS315TEF-tHMG1(ABE 4740) was excised using *BamHI* and *SalI* and then cloned in vector pRS315GPD CRTI_A393T (ABE 5245) at using *BamHI* and *SalI* .
pRS416GPD-PS(At)	ABE 5809	*At*PS (phytoene synthase) from *Arabidopsis thaliana* from pRS416TEF-PS(At) (ABE 4159) was excised using *BamHI* and *XhoI* and then cloned in the vector pRS416GPD (ABE 5800) at *BamHI* and *XhoI* site.
pRSII313	ABE 5812	The CEN-vector bearing HIS3 marker and TEF promoter-MCS-terminator for yeast expression and Amp^r marker for selection in *E. coli* [30].
pRS414-GAL1-SiTPS	ABE 5818	SiTPS gene from pUC57-SiTPS (ABE 5789) was excised using *BamHI* and *XhoI* and then cloned in the vector pRS414-GAL1-GCN4 (ABE 2152) at *BamHI* and *XhoI* site.
pRS416-GAL1-SiCPS	ABE 5819	SiCPS gene from pUC57-SiCPS (ABE 5687) was excised using *Eco*RI and *XbaI* and then cloned in the vector pRS416-GAL1 (ABE 449) at *Eco*RI and *XbaI* site.

pRS414GAL1	ABE 5820	Vector was constructed by excising 550 bp of the insert from pRS416-GAL1 (ABE 449) using *SacI* and *XhoI* and then cloned in the vector pRS414-GAL1-GCN4 (ABE 2152) at *SacI* and *XhoI* site.
pRS414GAL1-SsSS	ABE 5821	SsSS gene from pRS416TEF-SsSS (ABE 5624) was excised using *XhoI* and *BamHI* site and then cloned in vector pRS414-GAL1-GCN4 (ABE 2152) at *XhoI* and *BamHI* site.
pRS416GAL1-CcCLS	ABE 5822	CcCLS gene from pRS313TEF-CcCLS (ABE 5623) was excised using *EcoR*I and *XbaI* site and then cloned in vector pRS416-GAL1 (ABE 449) at *EcoR*I and *XbaI* site.
pRS313-GPD	ABE 5823	Vector was constructed by excising GPD promoter using *Sac*I and *Xba*I site from pRS426GPD (ABE 446) and cloned at pRS313-TEF(ABE 3569) vector at *Sac*I and *Xba*I site.
pRSII313-GPD	ABE 5824	Vector was constructed by excising GPD promoter using *Sac*I and *Xba*I site from pRS426GPD (ABE 446) and cloned at pRSII313 (ABE 5812) vector at *Sac*I and *Xba*I site.
pRSII313-GPD-CYC	ABE 5825	Vector was constructed by excising CYC terminator using *Kpn*I and *Xho*I site from pRS416TEF-SsSS (ABE 5624) and cloned at pRSII313-GPD (ABE 5824) vector at *Kpn*I and *Xho*I site.
pRSII313-TEF-CYC	ABE 5826	Vector was constructed by excising TEF promoter using *Sac*I and *Xba*I site from pRS313TEF (ABE 3569) and cloned at pRSII313-GPD-CYC (ABE 5825) vector at *Sac*I and *Xba*I site.

2.3 OLIGONUCLEOTIDES

The list of various oligonucleotides used in this study is given in Table 4.

Table 4: List of various oligonucleotides used in this study

Sr. no	Primer name	Sequence (5'-3')
1	BPT1a	TGGTTAATTTTAAAGAGATCATAAAGAAAATTTTGTCTAAAAAAGATTAATATAAAGACAATAGCTTCAAAATGTTTCTACTCCT
2	BPT1rev	GACCGTACATATTGCAGCGCATTAAATGTTCTATATCTTCATATATAATAATCAATGTCAGCAAATTAAAGCCTTCGAGCGTCCC
3	BPT1c	TGCGCATGTTTCGGCGTTCGAAACTTCTCCGCAGTGAAAGATAAATGATCTAGCAACAATGAATACTAAAGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAAC
4	BPT1d	GTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACTTTAGTATTCATTGTTGCTAGATCATTTATCTTTCACTGCGGAGAAGTTTCGAACGCCGAAACATGCGCA
5	BPT1e	TGCCCTCAAATTTTTGGCCG
6	BPT1f	TCAACCACATTCGCAGTTGC
7	BPT1g	CAAATAAGTATATGGTTCAGTCAAAACT
8	BPT1h	TGTCACGCTTACATTCACGCC
9	METa	TCAAGTTCGTACATTTTTTGAAGCGTGTTGGACGGGACAGGTTGATTACATTTTTTAAACATAGCTTCAAAATGTTTCTACTCCT
10	METb	AGGAGTAGAAACATTTTGAAGCTATGCAAATTAAAGCCTTCGAGCGTCCC
11	METc	GGGACGCTCGAAGGCTTTAATTTGCATAGCTTCAAAATGTTTCTACTCCT
12	METrev	TTATGTATACGTTAAATATTACATTATTTTCGCATTTTTGTATTTATATTCAGTGAAATAGCAAATTAAAGCCTTCGAGCGTCCC
13	METe	TGCGCATGTTTCGGCGTTCGAAACTTCTCCGCAGTGAAAGATAAATGATCGCTAGATTCATTTACGATATGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAAC

Sr. no	Primer name	Sequence (5'-3')
14	METf	GTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACATATCGTAAATGAATCTAGCGATCATTTATCTTTCACTGCGGAGAAGTTTCGAACGCCGAAACATGCGCA
15	METg	GCCTCGTAAGTTCCTCCTGG
16	METh	TACTGCCGGGAACTGTGTTC
17	METk	TTCACATCAAGGTCATAAGAAG
18	METl	TGTCTGTTTTGTTCGGTTTGAT
19	Primer 6005	GATCATTTATCTTTCACTGCGGAGAAG
20	Primer 6006	GTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTC
21	TEFfw	GAGCTCATAGCTTCAAAATGTTTCTAC
22	cyc rev	GGTACCGGCCGCAAATTAAAGCCTTCG
23	GPD_vec rev	ATAAACTGAGCTCCAATTCGCCCTATAGTG
24	cyc-vector for	AAGGCTTTAATTTGCGGCCGGTACCAGCTT
25	TEF_vec rev	TGAAGCTATGAGCTCCAATTCGCCCTATAG
26	GPD for	GAGCTCAGTTTATCATTATCAATACTCGCC
27	40BP	AATTAACCCTCACTAAAGGGAACAAAAGCTGGTACCGGCC
28	BPF	AATTAACCCTCACTAAAGGGA
29	Xma1 GPD-F	ATACACCCGGGGAGCTCAGTTTATCATT
30	BPF Apa1 GPDrev	ACATAGGGCCCAATTAACCCTCACTAAAGGGA
31	RtGGPPS_F	TCTAGATGTCTTTGGATTGGTATGA
32	RtGGPPS_R	GGATCCTTAAACTTTTGGCAATTCA
33	NMet12 for	TCAAGTTCGTACATTTTTTGAAGCG
34	NMet12 rev	TTATGTATACGTTAAATATTACATTATTTT

2.4 MEDIA

All the media, buffers and stock solutions were prepared using Millipore elix 5 deionized water unless otherwise mentioned. They were sterilized, as recommended, either by autoclaving at 15 lb/inch^2 (psi) pressures at 121ºC for 15 minutes, or by using membrane filters (Advanced Micro devices Pvt. Ltd., India) of pore size 0.22 µm (for heat-labile compounds). Additional amino acid and nutrients were prepared as sterile stock and added as per requirements. Agar was added, if required, at a final concentration of 2.2%. Ampicillin was added at a final concentration of 100 µg/ml.

2.4.1 LB (Luria-Bertani) Medium (per 1000ml)	a) Yeast extract 5g b) Trypton 10g c) NaCl 10g pH of the above medium was adjusted to 7.0 with 1N NaOH and volume was made up to 1000 ml. Agar was added, if required, at a final concentration of 2.2%. Ampicillin was added at a final concentration of 100µg/ml (after autoclaving of the medium) for the selection of recombinant clones and their routine growth.
2.4.2 YPD (Yeast extract-Peptone-Dextrose) Medium (per 1000 ml)	a) Yeast extract 10g b) Peptone 10g c) Dextrose 10g Agar was added, if required, at a final concentration of 2.2%.

2.4.3 SD (Synthetic defined) Medium (per 1000 ml)	a) YNB 1.7g (Yeast Nitrogen Base without ammonium sulphate) b) Ammonium Sulphate 5 g c) Glucose 20 g d) Aminoacids 80 mg

2.5 BUFFERS AND STOCKSOLUTIONS

2.5.1 Ampicillin Stock Solution (100mg/ml)

The required amount of ampicillin (sodium salt) (10 g for 100 ml) was dissolved in the required volume of deionized water, and it was filter-sterilized using a 0.2 μm filter membrane. It was stored at -20°C in aliquots in micro centrifuge tubes.

2.5.2 50% Glycerol

It is used for preparing –80°C stocks of *E.coli* harboring desired plasmids. 50 mL of glycerol is dissolved in 50 mL of deionized water and mixed properly. The solution was autoclaved and stored at room temperature.

2.5.3 25% Glycerol

It is used for preparing –80°C stocks of yeast strains. 25 mL of glycerol is dissolved in 50 mL of deionized water and mixed properly. The solution was autoclaved and stored at room temperature.

2.5.4 Alkaline Lysis Buffers (Plasmid DNA preparation from *E.coli*)

a) Solution-I (Resuspension Solution)	a) 50 mM Glucose b) 25 mM Tris-HCl (pH 8.0) c) 10 mM EDTA (pH 8.0) Autoclaved and stored at 4ºC.
b) Solution-II (Lysis Solution) (freshly prepared)	a) 0.2 N NaOH (freshly diluted from a 10 N stock) b) 1% SDS (freshly diluted from a 10% stock) Stored at room temperature.
c) Solution-III (Neutralization Solution)	a) 5 MPotassium acetate 60 ml b) Glacialaceticacid 11.5 ml c) Deionizedwater 28.5 ml The resulting solution is 3 M with respect to potassium and 5 M with respect to acetate. It was stored at 4ºC.
d) TE Buffer (Tris-EDTA) (pH 8.0)	10 mM Tris-HCl(pH 8.0). 1 mM EDTA (pH 8.0).
e) TE-RNAse (stock prepared at 10 mg/ml)	Working stock 20 µg/ml in TE Buffer, pH 8.0.
f) PCI (Phenol-chloroform-isoamyl alcohol) Solution (100ml)	a) Phenol 50 ml [Equilibrated with Tris-HCl (pH 7.6)] b) Chloroform 48 ml c) Isoamylalcohol 2 ml Stored at 4°C in a dark brownbottle.

2.5.5 Agarose Gel Electrophoresis Reagents

a) 1× TAE (Tris-acetate-EDTA) Buffer (per 1000ml) (prepared from 50× TAE stock)	40 mM Tris-acetate. 1mM EDTA (pH 8.0). Autoclaved and stored at room temperature.
a) Orange-G dye (Gel loading dye, 6X)	0.25% orange-G and 30% glycerol
c) 0.7 - 1% Agarose gel in 1× TAE	
d) Ethidium Bromide (10 mg/ml)Stock	The final working concentration used at 0.5 µg/ml.

2.5.6 Solutions for preparation of Chemical competent *E. coli* cells[28][29]

LB media	250ml
Calcium chloride solution	0.1 M in 15% glycerol
DH5α	Overnight grown culture, without antibiotic

2.5.7 Yeast Transformation Solutions (*S. cerevisiae*) [33]

a) 10X Lithium acetate (per 1000 ml) (pH7.5)

100.2 g of lithium acetate dissolved in 900mL of deionized water. pH adjusted to7.5 with diluted glacial acetic acid and volume bought upto 1000mL. Autoclaved and stored at room temperature. It is autoclaved and stored at RT.

b) 10X Tris-EDTA(TE) (per 1000 ml) (pH7.5)

15.759 g of Tris-Cl and 2.92g of EDTA added to 800mL of deionized water.

pH adjusted to 7.5 and volume bought upto 1000mL.Autoclaved and stored at room temperature. It is autoclaved and stored at RT.

c) 50% PEG (Polyethylene glycol) (per 1000 ml)

500g of PEG3500 dissolved in 500mL of deionized water by vortexing. Volume made upto 1000mlby adding deionized water. It is autoclaved and stored at RT.

2.5.8 Breaking Buffer (for plasmid / genomic DNA isolation fromyeast)

a) 10 mM Tris-HCl (pH 8.0)

b) 1 mM EDTA (pH 8.0)

c) 100 mM NaCl

d) 1% SDS

e) 2% Triton X-100

2.5.9 Solutions for Hydroxylamine mutagenesis [34]

NaOH	90mg
HydroxylamineHCl	350 mg

Dissolved in 5 ml cold water and pH adjusted to 6.5. The solution was made fresh just before use.

SECTION B: METHODS

2.6 Growth and maintenance of bacteria and yeaststrains

The *Escherichia coli* strains DH5α was routinely grown at LB medium at 37°C. *E. coli* transformants were selected and maintained on LB medium supplemented with ampicillin.

The *S. cerevisiae* strains were regularly maintained on YPD medium and grown at 30°C. The yeast transformants were selected and maintained on SD medium with the supplement as perrequirements.

2.7 Recombinant DNA methodology (restriction digestion, ligation, the transformation of *E. coli,* PCR amplification, etc.)

All the molecular techniques used in the study for manipulation of DNA, bacteria, and yeast were according to standard protocols (Guthrie and Fink, 1991; Sambrook and Russell, 2001) or as per manufacturers' protocol unless specificallymentioned.

2.8 Transformation of yeast

The transformation of *S. cerevisiae* strains was carried out by the lithium acetate method[33]. *S. cerevisiae* cultures were grown in YPD at 30°C with shaking for 16-24 hrs and then reinoculated in fresh YPD to an initial OD_{600} of 0.2; cells were allowed to grow at 30°C for 4-5 hrs with shaking. Cells were harvested at 6000 rpm for 5 min, then were washed with sterile water followed by subsequent wash with 0.1 M lithium acetate solution (prepared

in TE, pH 7.5) and were finally resuspended in the same solution. Cells were incubated at 30°C for 30 min with shaking. The cells were spun down, suspended in 0.1 M lithium acetate solution to a cell density of 1×10^9 cells/ml and divided into 100µl aliquots. Approximately 50 µg (5µl of 10mg/ml stock solution) of heat denatured, salmon sperm carrier DNA, followed by 0.3 µg- 0.7 µg of plasmid/DNA fragment were added to each aliquot, and the whole cell suspension was incubated at 30 °C for 30 min. After the incubation, 0.3 ml of 50% PEG 3350 (prepared in 0.1 M lithium acetate, pH 7.5) was added to each tube, mixed well and again kept at 30°C for 45 min. The cell suspensions were subjected to heat shock at 42°C for 10 min. and the cells were allowed to cool to room temperature. The cells were pelleted down at 7000 rpm for 3 min. The cell pellet was resuspended in sterile water, and the appropriate volume of cell suspension was plated on selectionplates.

2.9 Growth assay by dilution spotting

For dilution spotting, *S. cerevisiae* strains carrying different plasmids were grown overnight in minimal media supplemented with appropriate amino acids. Primary cultures were grown for 12-16 hours at 30°C, with shaking at 200 rpm. Primary cultures were used to inoculate a secondary culture in the minimal selective medium for 8 hours or till OD_{600} reaches between 0.6-0.8 at 30°C. Cells were pelleted, washed with sterile water and resuspended into sterile water at OD_{600}= 0.2. Cells were then harvested and washed with deionized water and resuspended in water to make dilutions of different OD_{600}: 0.2, 0.02, 0.002, 0.0002. 10µL of different dilutions were spotted on SD plates supplemented with appropriate amino acids.

2.10 Invitro Hydroxylamine mutagenesis [34]

Approximately, 10 µg of plasmid DNA was dissolved in 0.5 ml of Hydroxylamine solution (90 mg NaOH, 350 mg hydroxylamine HCl in 5 ml

water, pH around 6.5. freshly made up before use). This mixture was incubated at 37°C for 20 hrs, and the DNA was purified using a Qiagen column. Finally, the pool of mutagenized plasmid was directly transformed into the appropriate yeaststrain.

2.11 Genomic DNA isolation from *S. cerevisiae*

Genomic DNA from *S. cerevisiae* strains was isolated as described in [29] using the glass bead lysis method and the breaking buffer, described in section 2.5.7. Selected yeast transformants were inoculated in 20 ml of selection medium, and the cultures were incubated at 30^{o}C with shaking for 18-20 hr. After the incubation, the cells were harvested at 5,500 rpm for 5 min at room temperature, and the pellets were suspended in 200 µl of STES lysis solution. Equal amounts of sterile, acid-washed glass beads (425 to 600 µm, Cat# G8772, Sigma) were added, and the cell suspensions were vortexed vigorously for 1 min at room temperature. The lysed suspensions were then treated with phenol-chloroform adding 300 µl of the phenol-chloroform solution and then vortexed for 1-2 min at room temperature. Then 200 µl of 1X TE was added, and the lysates were spun down at 15,000 rpm for 5 min at RT, and the aqueous phase was collected in a fresh microcentrifuge tube. Then 900 µl of absolute ethanol was added in the aqueous phase, and it was pelleted down at 15,000 rpm for 5 minutes. Then again the pellet was washed with 900 µl of 70% ethanol and centrifuged at 15,000 rpm for 5 minutes. After that supernatant was discarded and the tube was kept at 55°C for 20 minutes (ethanol should evaporate). Then the pellet was eluted in 20 µl of 1X TE and 30 µl of TERNAse and kept at 37°C for 45 minutes.

2.12 Plasmid isolation from *E.coli*

The cells were grown in LB medium having ampicillin antibiotic for 14-16 hrs. Centrifugation was performed at 6000 rpm for 5 minutes, and the supernatant was

discarded. 100 µl Solution 1 was added followed by gentle mixing and incubation in ice for 5 minutes. After that 200µl solution, 2 was added and gently mixed by reverting the microcentrifuge tube and immediately 150µl solution 3 was added by mixing and kept in ice for 10 minutes. The cells were centrifuged at 11,000 rpm for 10 minutes, and the supernatant was collected. An equal volume of phenol-chloroform-isoamyl was added which was mixed by vortexing for 2 minutes, and centrifugation was performed at 13,000 rpm for 5 minutes. The aqueous phase was collected, and an equal volume of isopropanol was added followed by incubation at room temperature for 15 minutes. Centrifugation was performed at 13,000 rpm for 10 minutes, and the pellet was resuspended in 70% ethanol. Centrifugation was performed at 10,000 rpm for 5 minutes, and the supernatant was discarded followed by incubation at 37^{o}C for 30 minutes. The pellet was resuspended in TE-RNAse, and the plasmids were stored at -20^{o}C.

2.13 Transformation of *E.coli*

E. coli competent cells were prepared using the calcium chloride method in accordance with standard protocols[32]. Transformation was carried out by adding plasmid or ligation mixture to the competent cells, incubated, followed by a thermal shock at 42 °C for 1 minute. Transformed cells were incubated in LB at 37 °C for 45 min, and plated on LB medium containing ampicillin.

Chapter 3

Results and Discussion

3.1 Selection of non-essential loci and host strain for integrating lycopene biosynthesis genes at multiple loci in *S. cerevisiae*

For the genomic integration of three carotenogenic genes namely RtGGPPS, RtCRT1, and AtPS in budding yeast, we have modified and further optimized the protocol by (Mans, R. *et.al.*) for gene editing using *in vivo* assembled plasmids containing single gRNAs. The host strain selected for the genomic integration shares the CEN.PK genetic background[35] and has *cas9* endonuclease already integrated into the genome[IMX672 (Y40595) - MATa ura3-52 trp1-289 leu2-3,112 his3 can1::*Sp*cas9-natNT2] was obtained from Addgene. The *cas9* is flanked by the TEF1 promoter and the CYC1 terminator and is equipped with the SV40 nuclear localization signal for targeting to the nucleus[21][25][36].

Two genetic loci are selected for the genomic integration of the carotenogenic genes to produce lycopene. BPT1 locus is selected for the integration of RtGGPPS gene, and MET12 locus is selected for the integration of (AtPS +RtCRT1) cassette. BPT1 is Bile pigment transporter 1 which is an ABC type transmembrane transporter of MRP/CFTR family and found in the vacuolar membrane[37]. It is located on chromosome XII, and its disruption does not cause loss of cell viability. The MET12 gene is located on chromosome XVI and encodes a protein that has MTHFR activity *in vitro*. Its null mutant has no phenotype and is prototrophic for methionine[38].

3.2 Assembling of other genetic elements for efficient markerless genomic integration of the carotenogenic genes by CRISPR

For the genomic integration by CRISPR-Cas9 system, three genetic elements are required – Cas9, gRNA and repair fragment which constitutes the gene of interest or recombinant cassette that has to be integrated at a particular locus. In the present work, gRNA was selected using the Yeastriction tool at http://yeastriction.tnw.tudelft.nl. Yeastriction tool extracts all possible Cas9 target sequences (20 bp followed by NGG) from a specified ORF and its complementary strand by discarding sequences containing six or more Ts can terminate transcription[39], and also the sequences were tested for off-targets[40].

The targets are ranked based on the presence of restriction sites (1 for containing and 0 for lacking a restriction site), AT content (1 having the highest AT content and 0 for the lowest AT content) [41] and secondary structure (1 having the lowest amount of pairing nucleotides and 0 for the highest number of nucleotides involved in secondary structures, indicated by brackets)[42].

By using the above tool, we designed primers specific to BPT1 and MET12 locus. The primers include the specific primers that can be annealed to form (i) a double-stranded repair fragment (ii) a double-stranded insert which contains the target sequence for the gene of interest. The tool is also helpful in designing two diagnostic primers that can be used to amplify the region surrounding the coding region of the target gene to confirm genomic integration.

Using the above primers (sequence in Table 3), PCR amplification was done to amplify the following fragments. The first being the linearized plasmid backbone fragment which was obtained by PCR using the selected single gRNA plasmid as a template:

A. pMEL16 with *HIS3* selection marker as a template for the integration of RtGGPPS at BPT1 locus. The size of the amplified band was ≈ 5.5 Kb.
B. pMEL17 with *TRP1* selection marker as a template for the integration of (AtPS +RtCRT1) cassette MET12 locus. The size of the amplified band was ≈ 5.5 Kb.

Secondly, ds-gRNA insert obtained by mixing two complementary single-stranded oligos in a 1:1 molar ratio followed by heating at 95^{o}C and then cooled at room temperature. The resulting gRNA fragment contained the 20 bp gRNA recognition sequences (specific to BPT1 and MET12 locus respectively), flanked by 50 bp overlaps with the linearized plasmid backbone.

The repair fragment accounts for the third fragment, which was obtained by PCR in such a way that the resulting PCR product is flanked by 60 bp sequences homologous to the up and downstream regions of the DSB break, allowing for effective repair by the HR-machinery.

A. For RtGGPPS gene, the size of the repair fragment is 1.9 kb.
B. For (RtCRT1+AtPS) cassette, the size of the repair fragment is 4.1 kb.

Genomic integration was aimed by co-transformation of the plasmid backbone, the ds-gRNA insert, and the repair fragment discussed above. After that, the transformants are selected on non-selective media (YPD) for CRISPR plasmid removal (vector backbone + ds gRNA insert) for 5-6 rounds of selection. The desired genetic modification was checked initially by genomic DNA isolation followed by diagnostic PCR and later, sequencing of the putative positive clones for the confirmation of the correct genetic modification.

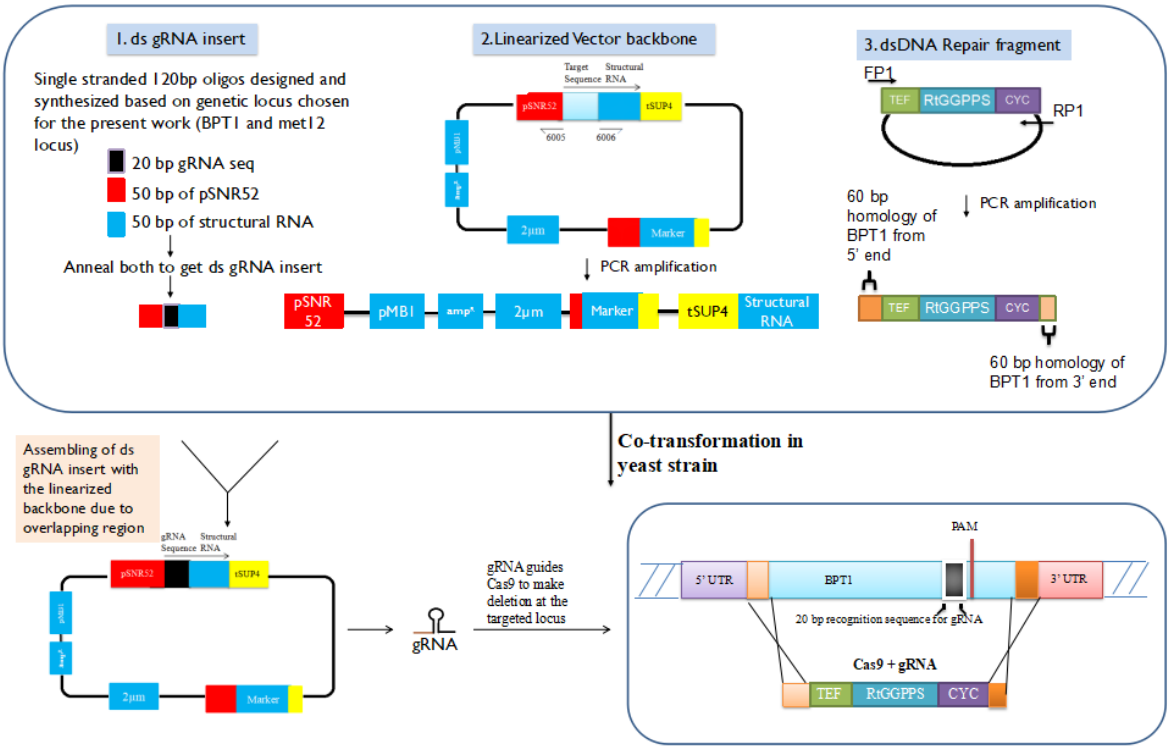


Figure 9. Schematic representation of the workflow for genomic integration of RtGGPPS at the BPT1 locus by CRISPR-Cas9 system in *S. cerevisiae*

3.3 Evaluating parameters critical for genomic integration of RtGGPPS

1) Amount of repair fragment

Initially, the yeast transformation efficiency was very low. Then the amount of repair fragment was increased for better transformation efficiency (from 250 ng to 5 µg) as many reports suggest that at least five µg of template DNA

is needed per yeast transformation for correct genetic modification[28]. This resulted in better transformation efficiency. However, even with increased transformation efficiency, there was no success in obtaining any positive results.

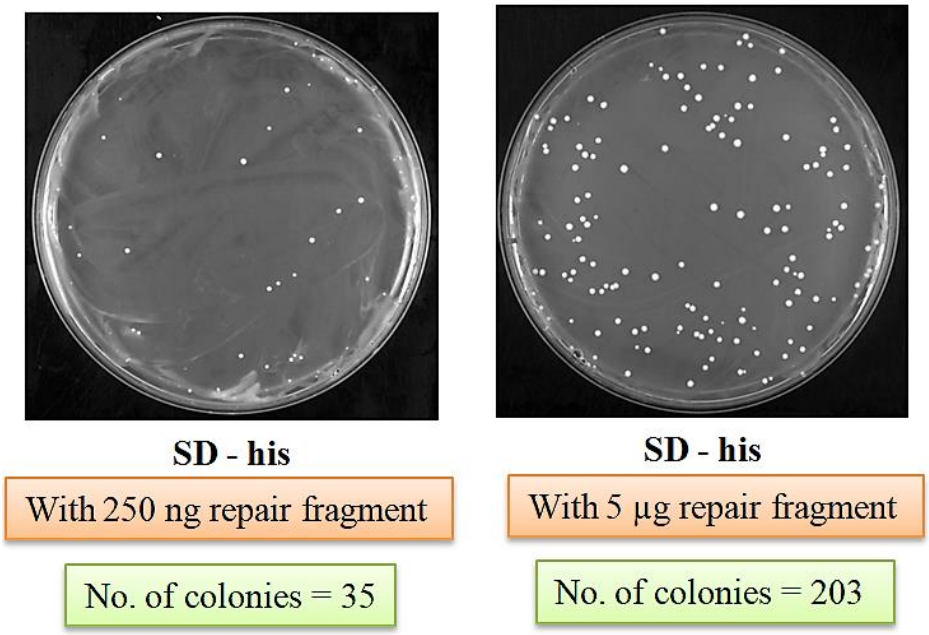


Figure 10. Transformation efficiency for the integration of RtGGPPS at BPT1 locus with different amount of repair fragment

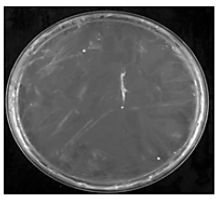
2) gRNA efficiency

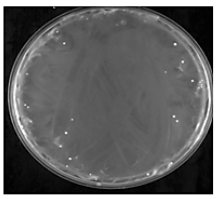
Another important aspect which has been repeatedly pointed out in previous reports was gRNA efficiency. First, we checked the gRNA chosen by us (ranked 1 by Yeastriction tool) in other software like 'CHOPCHOP' and 'CRISPy.' However, we found that the same gRNA is not ranked 1 there means the selection criteria of gRNA by Yeastriction and other software tools may not be an absolute one. Therefore, experimental verification of its efficiency is needed. For this, three sets of yeast transformation were done to check the gRNA efficiency in case of BPT1[28].

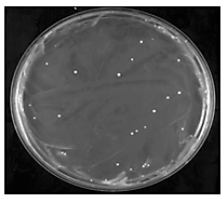
The first set was the negative control that has WT yeast strain only, and it was

selected on the non-selective media so there should be no colonies in this set of transformation.The second set was transformed with ds-gRNA insert and the linearized backbone in the yeast strain so that it would make gRNA and make disruption at the targeted locus. When the CRISPR plasmid was introduced without a repair template, it will repeatedly cleave the target locus, causing toxicity. Very few or no colonies are the ideal outcome since this indicates highly efficient CRISPR cleavage and low background rate. Cells can survive the CRISPR plasmid uptake without repair DNA if the CRISPR activity is stochastically low (such as due to poor gRNA efficiency) or mutations at the CRISPR target locus can be tolerated (which produces false transformants even in the presence of the repair template). Finally,the third set was transformed with ds-gRNA insert and the linearized backbone in the yeast strain along with the repair fragment; the resulting transforming colonies will be the correct transformants which bear the sequence of the repair template or the false transformants which bear the original, unedited sequence.

The experiment was done in triplicate, and the results were comparable. Details of the experiment are explained below.


SD - his


SD - his


SD - his

	Set 1	Set 2	Set 3
CRISPR-Cas9 vector	-	+	+
Repair fragment	-	-	+
Expected no.of Transformants	none	None (due to DSB lethality)	large no. of colonies
Observed no.of TransformantS	3	34	133

gRNA is moderately efficient

Figure 11. Assay to check the gRNA efficiency for the integration of RtGGPPS gene at BPT1 locus

For the above sets of transformation, we got three colonies in set 1 that were the false transformants, 34 colonies in the 2nd set and 133 colonies in set 3 which showed that our gRNA is moderately efficient to guide Cas9 due to which the CRISPR activity is stochastically low.

3.4 Integration of RtGGPPS at BPT1 locus

In the event of BPT1 locus gRNA being moderately efficient, it would be difficult and time-consuming to find out positive transformants by doing repeated rounds of genomic DNA isolation and diagnostic PCR thereafter. There needs to be a visual screen which can preliminary screen for positive colonies. So, we designed a screening strategy. First, the recombinant plasmid containing the combined cassette (RtCRT1+RtPSY1) was transformed in the yeast strain due to which the positive transformants had light yellow color due to the formation of carotenoids. In the positive transformants that had (RtCRT1+RtPSY1) cassette,

yeast transformation was done to make the desired genetic modification. After that, the transformants that will have the integrated RtGGPPS gene will be orange in color due to the production of β-carotene. So, this will limit the pool of putative positive colonies as the orange coloniesto be picked for further screening.

On performing this experiment, 13 orange colored colonies were obtained out of which one colony did not survive upon CRISPR plasmid curing.

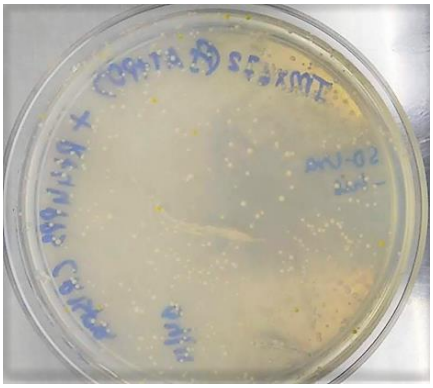


Figure 12. Transformants for genomic integration of RtGGPPS gene in the (RtCRT1+RtPSY1) cassette containing yeast strain

Yeast genomic DNA for the remaining 12 colonies was isolated. This was followed by diagnostic PCR using locus-specific primers. Initially, low colony diagnostic PCR efficiency was observed for the transformants. We, therefore, repeated the PCR by varying the concentration of the template DNA, but still, we were not able to get the amplified band (Fig.13 B). Since this could be due to the large band size (≈ 5.7 Kb), we designed new primers for the amplification of the region of interest such that amplified region includes the flanking region of the BPT1 locus and some part of the integrated RtGGPPS gene resulting in a shorter amplification to increase the PCR efficiency. Similarly, junction primers were made for the integration of (AtPS+RtCRT1) at MET12 locus.

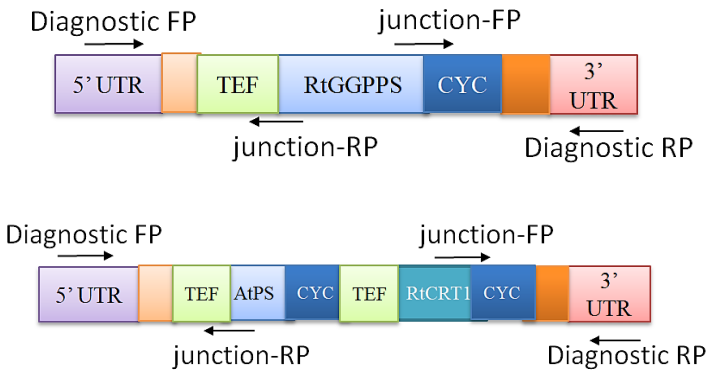


Figure 13. Designing of junction primers to check for the correct genetic modifications

We performed diagnostic PCR with two sets of primers 1) junction-specific 2) gene specific. Table 4 shows the expected amplified band size with all sets of primers in the case of WT and integrants.

Table 5: Expected band size in case of WT and integrants with locus-specific and gene-specific primers for the integration of RtGGPPS

Primers used	WT	Integrants
With D-FP and D-RP (locus-specific)	≈ 5.7 Kb	≈2.3Kb
With D-FP and junction-RP	No band	≈750 bp
With R-FP and R-RP (gene-specific)	No band	≈1.1 Kb

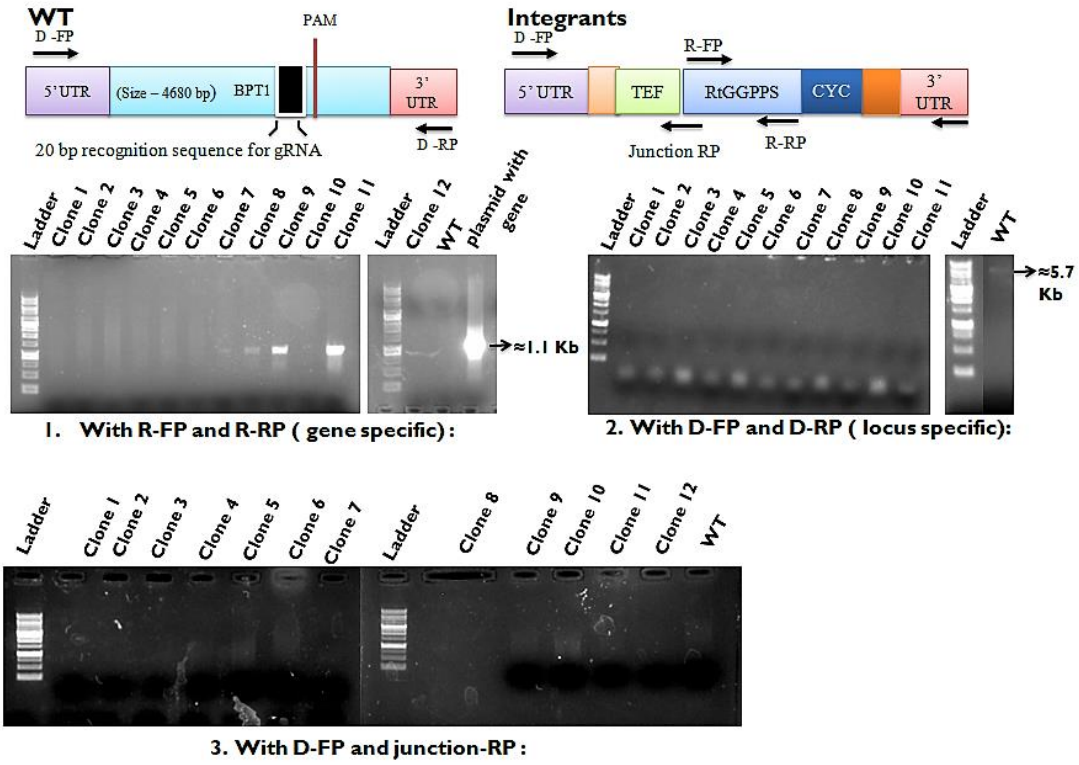


Figure 14. Diagnostic PCR of all the 12 clones with gene-specific, locus-specific and junction-specific primers for RtGGPPS integration

When the junction-specific primers were used, none of the transformants showed the band at ≈750 bp, and when the gene-specific primers were used, four clones (Clone 7, Clone 8, Clone 9 and Clone 11) showed band for the RtGGPPS gene. This implies that in these four clones, integration has occurred but not at the targeted locus. In these four clones, off-targeting occurred which means that the gRNA is not effectively efficient, i.e., it has high chances of off-targeting. This was checked by doing a BLAST analysis of the 20 bp gRNA sequence against the genome of CEN.PK113-7D strain and many off-targets were found.

3.5 Integration of (AtPS + RtCRT1) at MET12 locus

Since the gRNA efficiency is an important consideration, the gRNA selected was evaluated by BLAST analysis. In this analysis, the 20 bp gRNA sequence was taken and blasted against the genome of CEN.PK113-7D strain to identify the potential off-targets and the gRNA was found to be efficient. In clone seven that has RtGGPPS gene stably integrated into the genome, we

further transformed the (AtPS+RtCRT1) cassette to make the genetic modification at the MET12 locus. So, if the cassette is successfully integrated only then the color of the integrant will be orange. Thirty-three orange colored colonies were obtained. From all the orange colored colonies, CRISPR plasmid was cured, and then genomic DNA was isolated followed by the diagnostic PCR using two sets of primers 1) locus-specific 2) gene specific. Table 5 shows the expectedamplified band size with both sets of primers in the case of WT and integrants.

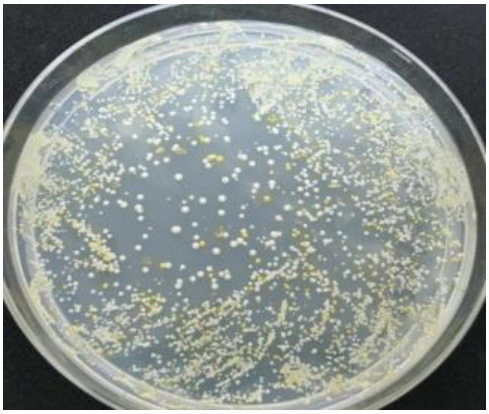


Figure 15. Transformants for genomic integration of (RtCRT1+AtPS) cassette in the RtGGPPS integrated yeast strain (clone 7)

Table 6: Expected band size in case of WT and integrants with locus-specific and gene-specific primers for the integration of (AtPS+RtCRT1)

Primers used	WT	Integrants
With D-FP and D-RP (locus-specific)	≈ 2.7 Kb	≈4.5 Kb
With P-FP and P-RP (gene-specific)	No band	≈1.1 Kb

When the locus-specific primers were used, all of the transformants showed band at ≈2.7Kb, and when the gene-specific primers were used, none of the transformants showed band at ≈1.1Kb. This implies that all of the transformants were negative.

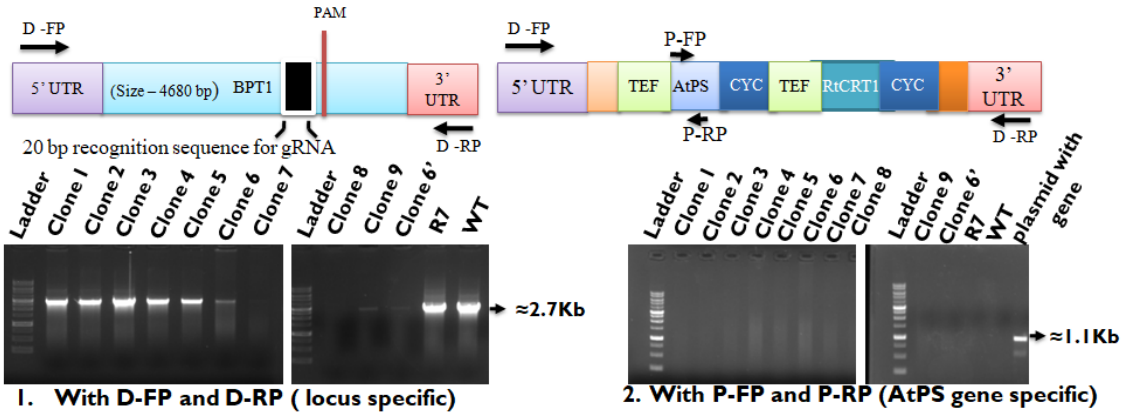


Figure 16. Diagnostic PCR of 10 clones with locus-specific and gene-specific primers for the integration of (AtPS+RtCRT1)

We found out that still, the efficiency of successful integration is low. Therefore, we need to screen more transformants.

3.6 Cloning of sclareol biosynthesis genes downstream of different strength of promoters

Since the window at which the substrate consumption assay will be functional was not known, we have expressed the sclareol biosynthesis genes under promoters of different strength. In addition, we have also expressed tHMG1 and AtPS genes under a stronger promoter.

Table 7: List of constructs made

GPD **p416GPD-CYC** **pRS313GPD-CcCLS-CYC** **p426GPD-SsSS-CYC** **pRS315GPD-tHMG1-CYC** **p416GPD-AtPS-CYC** **pRS313GPD-CYC** **pRSII313GPD** **pRSII313GPD-CYC**	**GAL1** **p414GAL1-SiTPS-CYC** **p416GAL1-SiCPS-CYC** **p414GAL1-CYC** **p414GAL1-SsSS-CYC** **p416GAL1-CcCLS-CYC**
TEF **pRSII313TEF-CYC**	**CYC** All the constructs under CYC promoter had been previously made in the lab

3.7 Discussion

Markerless genomic integration of the three carotenogenic genes needed for a functional substrate consumption assay based colorimetric screen was attempted by CRISPR-Cas9 mediated genomic integration strategy. Genomic integration of RtGGPPS gene at the unidentified locus was evident by diagnostic PCR results, however further optimization of the protocol is needed for stable on-site integration of the genes.

Various parameters, e.g., repair fragment amount,gRNA efficiency were found to affect transformation as well as targeting efficiency. However, further optimization needed to achieve stable genomic integration of the three genes. At present, an *S.cerevisiae* codon-optimized Cas9 is being attempted instead of human codon-optimized Cas9 variant Cas9 used by (Mans, R. *et.al.*) for markerless genomic integration. Also, another parameter, i.e., the length of the homologous arms of the repair fragment needs to be increased as it is critical for efficient replacement and being tried recently.

Although CRISPR-Cas9 system has made it possible to create mutations and gene editing in many organisms, markerless genomic integration of large recombinant gene cassettes at single or multiple loci is still challenging in yeasts as well as in other organisms. In the light of insufficiency of *in silico* based prediction of genome targeting efficiency using both gRNA efficiency and suitable genetic locus at which the gRNA would be targeted by the CRISPR-Cas9 system, in the present work we worked on a method that can be used to check genome targeting efficiency. In this method, the host strain is first transformed with a selectable plasmid carrying (RtCRT1+RtPSY1) and is followed by co-transformation by repair fragment and *in vivo* assembling gRNA plasmid. If RtGGPPS is integrated with high targeting efficiency at the chosen unidentified genetic locus, the percentage of yellow colored colonies will give the targeting efficiency. This low cost, visual screening plate-based method can be further developed and used in yeast as well as other organisms including humans to check gRNA as well as genetic locus suitability and compatibility for the process of targeted genetic modification specially markerless genomic integration. Constructs for substrate consumption assay for screening the diterpene synthase mutants in yeast were made
under different strength of promoters.

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